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HMGB1: REGULATION OF INFLAMMATORY FUNCTIONS AND THERAPEUTIC BLOCKADE

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HMGB1: REGULATION OF INFLAMMATORY FUNCTIONS AND THERAPEUTIC BLOCKADE

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If it was easy, everyone would be doing it

To my little family

ABSTRACT

The intracellular protein High Mobility Group Box Protein 1 (HMGB1) has been identified as a pivotal mediator of inflammation. HMGB1 can be released by various mechanisms and as an inflammatory mediator it induces both migration of inflammatory cells and cytokine production. Consequently, HMGB1 has been demonstrated to contribute to pathology in several inflammatory conditions. Increasing evidence indicate that HMGB1 post-translational modifications (PTMs) regulate both secretion and function of HMGB1.

The focus of this thesis work has been to investigate how selected PTMs regulate HMGB1 function and release and to define the presence of such modifications on HMGB1 in synovial fluid from patients with juvenile idiopathic arthritis (JIA). Furthermore, I have set the basis for HMGB1-blockade as a clinical treatment option by generating and characterizing the first known chimeric, humanized anti-HMGB1 antibody.

To examine the impact of redox-dependent PTMs on HMGB1 function, we first generated several cysteine redox isoforms of HMGB1. We found that all cysteines residues (C23, C45 and C106) required a defined redox state. A disulfide bridge between C23 and C45 with a concomitant C106 thiol was necessary for HMGB1 mediated cytokine-induction. In this disulfide redox isoform, HMGB1 activates TLR4. Furthermore, I have studied PTMs and their impact on HMGB1 secretion. We demonstrated that NLRC4 inflammasome activation induces hyperacetylation of key lysine stretches known to be associated with HMGB1 secretion, independently of priming signals. Addition of a priming signal induced reactive oxygen species (ROS) that stimulated a structural transition of HMGB1 to its cytokine-inducing, disulfide form. Hyperacetylated HMGB1 correlated significantly with inflammatory HMGB1 redox isoforms in joint fluid from JIA patients, indicating that HMGB1 is actively secreted during JIA and possesses inflammatory properties.

In addition, I recorded beneficial effects of mouse monoclonal anti-HMGB1 antibody (m2G7) treatment in experimental arthritis and in acetaminophen-induced liver injury. Importantly, I could demonstrate that a partly humanized version of the antibody (h2G7) retained its *in vitro* properties and *in vivo* therapeutic effects.

In conclusion, this thesis has significantly increased the understanding of the regulation of HMGB1 secretion and function during inflammation. The generation of an anti-HMGB1 chimeric antibody is an important step in development of a clinical anti-HMGB1 treatment.

LIST OF PUBLICATIONS

I. **Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1)**
Huan Yang, Peter Lundbäck, Lars Ottosson, Helena Erlandsson-Harris, Emilie Venereau, Marco E. Bianchi, Yousef Al-Abed, Ulf Andersson, Kevin J. Tracey, Daniel J. Antoine
Molecular Medicine. 2012 Mar 30;18:250-9

II. **TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis**
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EMBO Journal. 2013 Jan 9;32(1):86-99

III. **Characterization of the inflammatory properties of actively released HMGB1 in juvenile idiopathic arthritis**
Peter Lundbäck, Pernilla Stridh, Lena Klevenvall, Rosalind E. Jenkins, Marie Fischer, Erik Sundberg, Ulf Andersson, Daniel J. Antoine, Helena Erlandsson Harris
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IV. **Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models**
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V. **Successful therapeutic HMGB1 neutralization using a humanized, chimeric monoclonal antibody**
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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGEs	Advanced glycation end-products
AIM2	Absent in melanoma-2
ALR	AIM-like receptor
ALT	Alanine aminotransferase
ANA	Anti-nuclear antibodies
Anti-CCP	Anti–citrullinated protein antibody
APAP	Acetaminophen/Paracetamol
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
AUC	Area under the curve
BIR	Baculovirus inhibitor repeat
CAPS	Cryopurin-associated periodic syndromes
CARD	Caspase activation and recruitment domain
CBP	Calmodulin-binding protein
CD	Cluster of differentiation
CDAMPs	Cell-death-associated molecular patterns
CIA	Collagen-induced arthritis
CID	Collision-induced dissociation
CII	Collagen type II
CLR	C-type lectin receptors
CRP	C-reactive protein
CYP450	Cytochrome P450
DAMPs	Damage-associated molecular patterns
DTT	Dithiothreitol
E. coli	*Escherichia coli*
Fc	Crystallizable fragment
FcR	Fc receptor
GMP	Good manufacturing practice
GSH	Glutathione
HAMA	Human anti-mouse antibodies
HATs	Histone acetyl transferase
HDACs	Histone deacetylase
HIN	Hematopoietic interferon-inducible nuclear protein
HMGB1	High mobility group box protein 1
HSPs	Heat shock proteins
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILAR	International League of Association for Rheumatology
IP	Immunoprecipitation
IRFs	Interferon-regulatory factors
JAK	Janus kinase

JIA	Juvenile idiopathic arthritis
LDH	Lactate dehydrogenase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
mAbs	Monoclonal antibodies
MAS	Macrophage activation syndrome
MBL	Mannose-binding lectin
MD2	Myeloid differentiation factor-2
MHC	Major histocompatibility complex
MMP	Mitochondrial membrane potential
MSU	monosodium urate crystals
mtROS	mitochondrial ROS
MyD88	Myeloid differentiation primary response protein 88
NACHT	Nucleotide binding and oligomerization domain
NADPH	Nikotinamid-adenin-dinukleotidfosfat
NAIP	NLR apoptosis inhibitory protein
NAPQI	N-acetyl-para-benzoquinone imine
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NLR	Nucleotide–binding oligomerization domain-like receptors
NLS	Nucelar localization sequences
NOX	NADPH-oxidase
NSAID	Non-steroidal anti-inflammatory drugs
MMP-3	Matrix metalloproteinase 3
oxLDL	Oxidized low-density lipoprotein
pAbs	Polyclonal antibodies
PAMPs	Pathogen associated molecular patterns
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PTMs	Post-translational modifications
PYD	Pyrin domain
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycated end-products
RF	Rheumatoid factor
RIG-I	Retinoic acid-inducible gene 1
RLRs	RIG-like receptors
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
TBP	TATA binding protein
TIM-3	T cell immunoglobulin mucin-3

TIR	Toll/interleukin-1 receptor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRX	Thioredoxin

1 INFLAMMATION AND IMMUNITY

Inflammation is a biological response elicited against invading pathogens or tissue damage induced by chemical or mechanical insult. The inflammatory response is orchestrated by cells and soluble mediators of the innate immune system that initiate a complex cascade of events in an attempt to induce cleareance of pathogenic agents and damaged cell material and to restore tissue homeostasis. Acute inflammation is a pivotal part of an immune response against microbial infections and tissue injury. However, if inflammation remains unresolved and uncontrolled after removal of the inducing agent, the inflammation itself may cause tissue destruction and this is a typical hallmark of many inflammatory diseases.

1.1 The Immune system

The immune system can be divided into the innate and the adaptive immune systems and has evolved in order to defend the human body against invading pathogens and tissue trauma. Innate immunity acts as a first line of defense and includes anatomical barriers (physical and chemical) that prevent the entry and colonization of pathogens. If the barrier is breached, tissue resident innate immune cells and soluble mediators have the important role of discriminating autologous from non-autologous material and, in addition, recognizing tissue damage. Innate immune cells are equipped with germline-encoded pattern recognition receptors (PRRs) that survey intra- and extra-cellular compartments for highly conserved pathogen structures. These conserved pathogen structures, also known as pathogen associated molecular patterns (PAMPs), are normally critical for pathogen survival and are evolutionarily conserved. Activation of PRRs can also occur by interaction with self-molecules, known as damage-associated molecular patterns (DAMPs). DAMPs are defined as endogenous molecules that are abberantly expressed and released by damaged cells and in some cases also by activated and stressed cells. Activation of PRRs instigates an immune and inflammatory effector response in an attempt to induce pathogen clearance, removal of dead cells and restoration of tissue homeostasis [1].

The innate immune system provides an instant response against invading pathogens or tissue damage (within hours) and has a generic specificity defined from birth. Due to evolutionary pressure by the immune system some pathogens have evolved and aquired features that make them undetectable for the innate immune system. If the innate immune system fails to recognize or eliminate an infection the adaptive immune response will take effect (within days-weeks). The adaptive immune system primarily protects us against re-infections and has ‘endless’ antigen specificity and memory function, in contrast to the innate immune response. Activation of the innate immune system is also fundamental in conducting an appropriate adaptive immune response since the inflammatory mediators produced by the innate immune system can polarize and regulate subsequent adaptive immune cell responses [2].

1.2 Inflammation

Tissue trauma can be of either infectious or non-infectious origin and both induce inflammatory responses. The main goals of an inflammatory response are to eliminate the inducing cause, remove

damaged tissue and to initiate a repair process in order to restore tissue function. Stimuli that induce acute inflammation are pathogens, necrotic tissue and foreign substances that cause non-physiological tissue death.

Upon such stimulations tissue guardian cells (primarily resident phagocytic cells but also mast cells) will become activated and secrete inflammatory mediators in an attempt to initiate clearance of the inducing agent. These inflammatory mediators include cytokines and chemokines that have auto-, para- and/or endocrine function on other cells [3]. Within a couple of hours the release of these inflammatory mediators also induces recruitment of blood-borne leukocytes to the site of inflammation or tissue trauma via a process called extravasion. Independently of the initiating cause the activation of cells and release of inflammatory mediators give rise of the classical signs of acute inflammation: *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *functio laesa* (loss of function). Heat, redness and swelling are accounted for by an increased blood flow and endothelial leakage occurring within minutes after tissue injury. The pain alerts the host of an atypical state of the affected tissue and if the inflammatory reaction is dysregulated this can result in a loss of function. The inflammatory response is tightly controlled and mostly self-limited in order to avoid collateral tissue damage. An inappropriate and unresolved inflammatory response can lead to chronic inflammation. This may be induced by persistent infections, long-term exposure to immune irritants (silica or other adjuvant substances) or due to underlying autoimmunity disorders. In the latter, the host immunity has lost the control of sufficient self-tolerance [2].

1.2.1 Recognizing danger – innate immune sensors

The innate immune system detects a variety of pathogenic structures through both soluble and cellular pattern recognition receptors (PRRs). Acute phase proteins such as C-reactive protein (CRP) and mannose-binding lectin (MBL) are soluble PRRs that recognize pathogen specific carbohydrates and 'tag' pathogens as non-self, thus promoting phagocytosis and induction of complement-mediated attack. Cellular PRRs include toll-like receptors (TLRs), nucleotide–binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) [4]. TLRs and CLRs are mainly expressed on the membrane surface whereas the RLRs, ALRs, NLRs and some TLRs are expressed intracellularly. Ligation of PRRs by PAMPs or DAMPs in most cases initiates intracellular signaling cascades that result in the production of pro-inflammatory mediators and cellular activation functions such as phagocytosis. Conversely, activation of ALRs and some NLRs may cause the assembly and activation of a large caspase-1 activating multiprotein complex called the inflammasome. A pathogen or tissue injury can cause simultaneous activation of multiple PRRs and the sum of those triggering events defines the extent of the consequent immune response. Activation of PRRs can also induce the expression and activation of other PRRs, thus causing a chain-reaction of controlled inflammatory events. Inappropriate regulation of PRR activation can potentially lead to states of chronic inflammation and autoimmune diseases [2].

1.2.2 TLRs – The toll keepers of immunity

TLRs are evolutionarily conserved receptors that are homologues to the *Drosophila* Toll protein, whose immunological importance in microbial infections was first discovered in 1996 [5]. TLRs are predominantly expressed in tissues exposed to the outer environment and in tissues with prominent immune function. Both immune cells and non-professional immune cells express TLRs, including phagocytes, B cells, T cells, endothelial cells, epithelial cells and fibroblasts. TLRs make up a family of 10 transmembrane-spanning proteins (TLR1 to 10) in humans and of 12 in mice (TLR1 to 9 and 11 to 13). Many tissues express at least one TLR but the highest diversity of TLRs is predominantly expressed by professional phagocytic immune cells [6]. TLRs are structurally composed of leucine-rich repeats (LRR) at the exterior N-terminal domain and have a cytoplasmic domain that is homologous to that of the interleukin (IL)-1 receptor, thus termed the Toll/IL-1 receptor (TIR) domain. All TLRs contain several LRRs and the ligand specificity for PAMPs and DAMPs resides among these regions. Some TLRs have extracellular co-receptors or adapter molecules that confer both specificity and responsiveness [7, 8].

TLR signaling is initiated by a ligand-induced homo- or hetero-dimerization followed by recruitment of other intracellular TIR adaptor proteins via the TIR domain. Ligation of different TLRs activates distinct signaling pathways depending on the integrated adaptor proteins. TLR transduction can roughly be divided into myeloid differentiation primary response protein 88 (MyD88)-dependent or -independent signaling pathways. Several transcription factor families become activated downstream of TLR signaling and the two major ones are nuclear factor-kappa B (NF-κB) and the interferon-regulatory factors (IRFs). NF-κB is mainly activated by the MyD88-dependent pathway that is utilized by most TLRs, and the effects of this activation are diverse, resulting in up-regulation of immune stimulatory cell surface proteins and production of pro-inflammatory mediators such as tumor necrosis factor (TNF), IL-1β and IL-8. The MyD88 independent (or TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent) pathway activates IRFs in addition to NF-κB and induces the production of type I interferons (IFN) (mainly IFNα and IFNβ)[7].

In contrast to other TLRs, TLR3, 7, 8 and 9 are expressed in the endosomal compartment rather than in the outer cellular membrane and are mainly activated by viral or bacterial nucleic acids [1]. Signal transduction for TLR3 is mediated via the adaptor molecule TRIF and, consequently mainly leads to the production of type I IFNs that are crucial for anti-viral responses. In contrast, TLR7, 8 and 9 utilize the MyD88-dependent pathway and activates both NF-κB and IRF signaling. TLR4 is the only TLR that can utilize both MyD88 and TRIF [7].

1.2.3 Inflammasome activation - You Only Live Once

The NLRs constitute a protein family of intracellularly expressed sensor proteins that become activated by cytosolic PAMPs and DAMPs [9]. Mutations in several of the 22 known human NLR genes are linked to auto-inflammatory and autoimmune diseases, emphasizing the role of NLRs in inflammation [10, 11]. NLRs

contain three distinct domains including a C-terminal LRR domain that confers ligand sensing, a central nucleotide binding and oligomerization domain (NACHT), and an N-terminal effector domain. There are to date four known N-terminal effector domains and the subtype of the effector domain defines the subfamily of NLRs. NLRPs contain a pyrin domain (PYD), NLRCs a caspase activation and recruitment domain (CARD), NLRBs a baculovirus inhibitor repeat (BIR) domain and NLRA contains an acidic trans-activating domain [12-14] (**Figure 1**).

Activation of several NLRs results in oligomerization and formation of a large multiprotein inflammasome complex that have the capacity to activate inflammatory pro-caspases, especially autocleavage and activation of pro-caspase-1. The inflammasome complex is named after the component NLR. The NLRs lacking a CARD domain require the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which contains a PYD and CARD domain. Once caspase-1 has become activated, proteolytic cleavage and activation of pro-IL1β and pro-IL18 is performed and the cleaved proteins are subsequently released in their active forms. IL1β is a potent inflammatory mediator involved in both systemic and local inflammation is controlled on several levels. Inflammasome activation is also known to induce the release of DAMPs such as IL-1α [15], IL-33 [16] and HMGB1 [17-19]. In addition to activation and release of inflammatory mediators, inflammasome activation can in certain cell types lead to cell death if sufficiently triggered. This programmed inflammatory type of cell death is denoted pyroptosis and is mainly a feature of macrophage inflammasome activation. The pyroptotic event can be regarded as an attempt to amplify the immunological response [13]. In addition to NLRs, other non-NLR proteins can also induce assembly of the inflammasome, including the DNA sensor AIM2 [20] and the RNA virus sensor RIG-I [21].

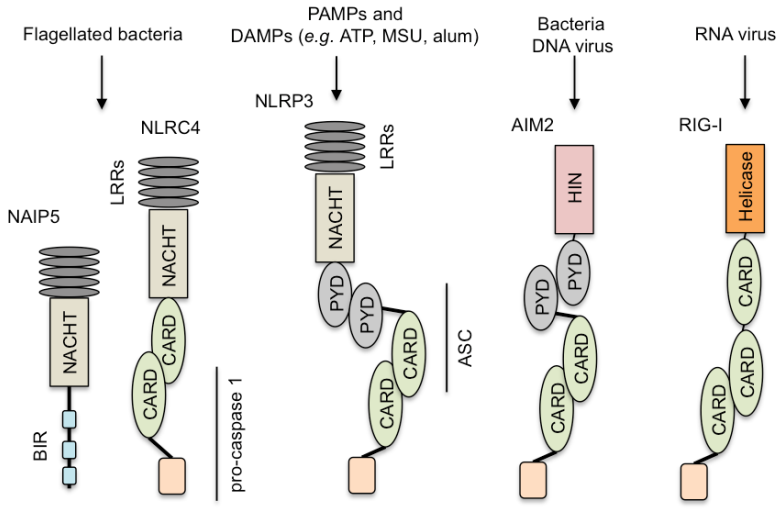

Figure 1: Structural domains of selected inflammasomes*. Abbreviation: HIN = hematopoietic interferon-inducible nuclear protein.*

There is an intricate interplay and crosstalk between different PRRs [22] and some require a signal from other PRRs in order to become fully activated. The NLRP3 inflammasome is the most studied inflammasome and requires two distinct signals to become activated. NLPR3 is unique among the NLRs in the sense that its low expression in resting cells is not sufficient for inflammasome activation [23, 24]. The priming signal (or the first signal) is usually the activation of a cell membrane-associated TLR or a cytokine receptor that results in increased expression of NLRP3 inflammasome components and pro-IL-1β, whereas pro-IL-18 is believed to be more constitutively expressed. The second signal promotes oligomerization and activation of the NLRP3 inflammasome complex and is usually provided by DAMPs or PAMPs such as extracellular adenosine triphosphate (ATP), monosodium urate (MSU) crystals, alum crystals or nigericin [25]. The NLRC4 inflammasome (encoded by *Ipaf* in mice) is activated by the cytosolic bacterial component flagellin [26] and, in contrast to the NRLP3 inflammasome, does not require a priming signal [27]. Flagellin induced NLRC4 inflammasome activation is assisted by the BIR domain containing NLR neuronal apoptosis inhibitory protein 5 (NAIP5) [28]. In addition, flagellin activates the cell membrane expressed TLR5, highlighting that PRRs act at different levels to promote an inflammatory response.

Increasing evidence has suggested a significant role of caspase-11 (humans caspase-4 or -5) in inflammasome activation and pyroptosis [19, 29, 30]. Initial studies and generation of *Casp1*$^{-/-}$ mice utilized strain 129 embryonic stem cells and since *Casp1* and *Casp11* is localized in close proximity in the genome, *Casp1*$^{-/-}$ mice lack both caspase-1 and caspase-11. This circumstance led to the discovery of non-canonical caspase-11-dependent inflammasome activation. In this sense caspase-11 acts upstream by activating components of the canonical pathways that induce activation of caspase-1 and maturation of IL-1β. Caspase-11 activation alone is sufficient to induce pyroptosis but not maturation of IL-1β [19, 31].

1.3 DAMPs

DAMPs are endogenous molecules that have the capacity to induce an inflammatory response in similar manner as PAMPs. An infection may result in the release of DAMPs and in such cases PAMPs and DAMPs can act in synergy to induce an inflammatory response. However, DAMPs may also be released by non-infectious events and can then alone initiate and perpetuate inflammation via PRR activation. The striking similarities in PRR activation, receptor sharing, signaling and subsequent effects may justify the analogous naming of PAMPs and DAMPs. However, the definition and nomenclature of DAMPs are still elusive and alternative names such as alarmins [32], hyppos [33], danger-associated molecular patterns and cell-death-associated molecular patterns (CDAMPs) [34] have been or are still in use. Alarmins would perhaps serve as the best definition since the word DAMP does not apply to larger immune-stimulatory multimolecular structures such as microparticles, nuclear extracellular traps (NETs) or even intact organelles, all of which contain multiple DAMPs [35-37]. In addition, stressed and immune-stimulated cells may induce the release of certain DAMPs without cellular damage or death. Independently of ambiguity of the nomenclature, the word DAMP will be used throughout this thesis.

Under normal conditions DAMP molecules are expressed intra- or extra-cellularly and exert functions that are distinct from their pro-inflammatory effects. Exposure to infectious or sterile agents can induce DAMP expression and release or a functional transition that can lead to sterile inflammation. Sterile DAMP inducers include trauma, ischemia, hypoxia, asbestosis exposure and toxic drug metabolites [38]. DAMPs that originate from the extracellular compartment are for example hyaluronan fragments [39], heparin sulfate [40] and oxidized low density lipoprotein (oxLDL) [41]. However, the classical view of a DAMP is an intracellular protein that becomes released either passively from dying cells or via non-classical protein secretion. Intracellularly expressed DAMPs include HMGB1, S100 proteins [42], heat shock proteins (HSPs) [43], ATP [44], IL-1α [45] and uric acid crystals [46]. Changes in the surrounding environment or molecular degradation are events that can potentially induce a pro-inflammatory transition. Once released and activated, DAMPs activate innate immune cells via PRRs either directly or indirectly. Some DAMPs also possess the capacity to restore tissue homeostasis by inducing reconstruction of the damaged tissue [35].

2 HMGB1

The protein HMGB1 has received a lot of attention over the past 15 years and is today considered as a prototypical DAMP. HMGB1 is an abundant intracellular protein that to a greater extent resides within the nucleus of most cells. HMGB1 can either be passively released as a result of cell death or alternatively secreted by activated immune cells such as monocytes, macrophages, dendritic cells, adenoid cells and natural killer (NK) cells [47-51]. Other non-immune cells including neurons, endothelial cells, smooth muscle cells and other cell types are also known to secrete HMGB1 [52-54]. HMGB1 function as a pleiotropic inflammatory mediator by activating several receptors.

2.1 Historical background

HMGB1 is a ubiquitously expressed protein present in most nucleated vertebrate cells with an extremely conserved sequence (99% between mammals). The name derives from its high mobility in gel electrophoresis [55] and the function of HMGB1 was first described as a regulator of DNA structure and transcription factor interactions. The 25kDa protein is structurally composed of two tandem DNA binding HMG domains (box A and box B) and a continuous stretch of acidic amino acids at the C-terminal domain (**Figure 2**). The HMG box family members are all capable of bending and regulating DNA structure by binding to the minor groove of DNA [56], and this is believed to be their main nuclear function. The binding of HMGB1 to DNA is very transient as compared to that of histones [57]. The highly conserved sequence, together with the fact that HMGB1-deficient mice die within 24h from birth highlight the importance of HMGB1 during development [58]. Other nuclear functions of HMGB1 include regulation of gene transcription. HMGB1 has the capacity to directly bind and affect nucleosome structure [59] and TATA binding protein (TBP) [60]. Furthermore, a direct interaction with certain transcription factors including members of the steroid hormone family [61], p53 [62], and NF-κB subunits [63] has been reported. HMGB1 is also known to regulate DNA repair processes by binding to damaged DNA [64]. Over the last 15 years significant attention has been focused on the fact that extracellular HMGB1 has pro-inflammatory effects distinct from its nuclear DNA binding properties.

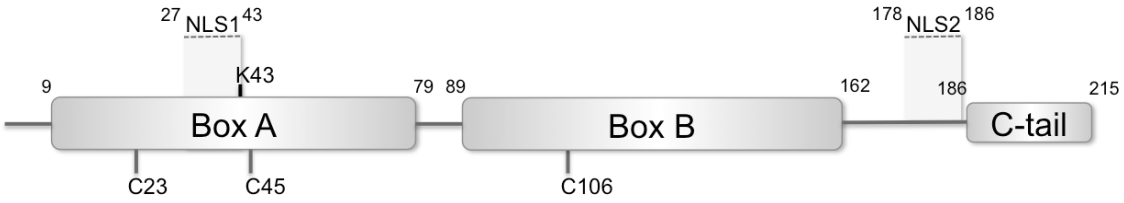

Figure 2: Overview of HMGB1 protein and its structural domains*. HMGB1 contains two DNA-binding domains (Box A and Box B) and an acidic C-terminal tail. The three conserved cysteine residues (C23, C45 and C106) are redox sensitive and dictate the inflammatory effects of HMGB1. For the cytokine-inducing effect of HMGB1 a disulfide bond between C23 and C45 and a simultaneous C106 thiol are required. For migration-inducing effect all cysteines require a thiol side-chain. Acetylation of lysines within the two NLS regions regulates the intracellular distribution of HMGB1 and is required for cytoplasmic accumulation and subsequent extracellular release. A mono-methylation of residue K43 is associated with HMGB1 release from neutrophils.*

The fact that HMGB1 has extracellular effects was first discovered in 1991. In that study, HMGB1 had significant effects on neurite outgrowth [65]. In 1999 Tracey and colleagues could demonstrate that HMGB1 was released with delayed kinetics when compared to TNF and IL-1β in experimental sepsis [66]. Delayed administration (up to 24h after induction) of anti-HMGB1 antibodies attenuated endotoxin lethality and was thus suggested to be a late mediator in the pathogenesis. Furthermore, HMGB1 was released with delayed kinetics in LPS-stimulated monocytes. Since the advent of these findings, increased levels of HMGB1 have been recorded in multiple diseases, as extensively reviewed in [67]. The extracellular functions of HMGB1 and its regulated release are the main focus of this thesis.

2.2 Regulation of HMGB1 release and inflammatory activity

Once transcribed and translated into protein HMGB1 undergoes extensive post-translational modifications (PTMs) including acetylation, methylation, phosphorylation, glycosylation and oxidation. During inflammation and inflammatory types of cell death HMGB1 can be released into the extracellular environment and initiate or perpetuate inflammation. Specific PTMs are known to regulate extracellular release of HMGB1 and also to modulate its inflammatory functions (**PAPERS I & II**).

HMGB1 is readily released from all damaged and necrotic nucleated cells in a passive manner. In contrast to necrotic cells HMGB1 binds strongly to chromatin in apoptotic cells and thus only leaks out in minute amounts [57]. If clearance of apoptotic cells fails or does not readily occur, the apoptotic cells undergo secondary apoptosis/necrosis and HMGB1 may be passively released. Several cell types possess the capacity to induce an active HMGB1 secretion without simultaneous cell death [50, 68]. However, significant HMGB1 levels released by active secretion are generally believed to be mainly derived from innate immune cells such as monocyte/macrophages, dendritic cells, natural killer (NK) cells or neutrophils.

Various inflammatory stimulators possess the capacity to induce active HMGB1 secretion. PRR activation by exogenous or endogenous stimulators can induce the release of HMGB1. In addition to stimulation by inflammatory mediators, changes in the physiological environment can also induce the secretion of HMGB1. Both hyperoxia [69] and hypoxia [70] have been demonstrated to induce the extracellular release of HMGB1, thus confirming the increased systemic HMGB1 levels recorded in ischemia-reperfusion injuries [71, 72]. Similar to the sepsis study reported in 1999 [66], HMGB1 seems to be a late mediator of disease and to perpetuate/aggravate inflammation when the stimulus is an inflammatory mediator activating via PRRs or cytokine receptors. In contrast, if the initiating trigger is environmental or caused by cellular trauma HMGB1 is likely to be an initiator of inflammation and could be considered as an early inflammatory mediator.

2.2.1 Cytoplasmic shuttling and extracellular release

HMGB1 is predominantly expressed in the nucleus of non-activated cells and as with other DNA-binding proteins, specific PTMs may alter its affinity to DNA. HMGB1 contains 43 acetylation-susceptible lysines

(20% of all amino acids). At least 17 can be acetylated *in vivo* [49] and mutually exclusive acetylation modifications would generate almost countless acetylation variants of HMGB1. However, two key lysine stretches are concomitantly acetylated. These stretches are non-classical nuclear localization sequences (NLS) and acetylation of these lysines tightly controls the nuclear-cytoplasmic shuttling of HMGB1 (from here on hyperacetylation will be used to refer to acetylation of all lysines within the NLS regions). Hyperacetylation of the NLS regions shifts the localization equilibrium of HMGB1 towards the cytoplasm by inhibiting re-entry to the nucleus [49]. Site-directed mutagenesis studies have demonstrated that lack of NLS region lysines retains HMGB1 within the nucleus [49]. Histone acetyl transferases (HATs) and histone deacetylase (HDACs) dynamically regulate the acetylation and deacetylation of histones and other nuclear proteins and are hence key in epigenetic control. The activity of HATs/HDACs also regulates the acetylation of HMGB1, and the nuclear/cytoplasmic shuttling and inhibition of HDACs promotes translocation and release of HMGB1 [49, 71].

In addition to hyperacetylation a mono-methylation of a lysine in position 43 (K43) induces the release of HMGB1 from neutrophils and is so far the only suggested cell type-specific PTM of HMGB1 [73]. K43 mono-methylation of HMGB1 lowers the affinity to DNA [73] and induces cytoplasmic accumulation in a similar way to acetylation in monocytes/macrophages [49]. The presence of acetylation PTMs in neutrophils are currently unknown. Likewise, phosphorylation of multiple serine residues (S35, S39, S42, S46, S53, and S181) has been suggested to induce cytoplasmic translocation of HMGB1 *in vitro* [74, 75]. Importantly, S35 is the only serine residue that has been reported phosphorylated *in vivo*, as recorded in serum from alcoholic liver disease patients and mouse serum [76]. Hence the concomitant phosphorylation of these serine residues with other PTMs and its relative contribution to cytoplasmic translocation and release of HMGB1 needs to be further elucidated.

HMGB1 lacks a leader peptide that normally determines the classic export route of extracellular proteins via the endoplasmic reticulum-GOLGI system. Cytoplasmic HMGB1 is instead packed into non-classical secretory vesicles in response to inflammatory stimuli [77]. The mechanism by which HMGB1 enters these vesicles is still unknown. The triggers that induce HMGB1 release are many, as previously mentioned. Interestingly, the kinetics of active HMGB1 release is usually delayed as compared to other pro-inflammatory mediators such as TNF or IL-1β [66, 77]. IL-1β is released in a similar way to HMGB1 by exocytosis of secretory lysosomes but with much earlier kinetics. This kinetic discrepancy is caused by a dependency on the formation of the bioactive lipid lysophosphatidylcholine (LPC), which occurs later in the inflammatory phase and which has been suggested to be pivotal for HMGB1 release [77]. The delayed kinetics of HMGB1 release are also likely dependent on the fact that HMGB1 needs to be translocated into the cytoplasm before entering secretory lysosomes. In contrast to HMGB1 release HDAC inhibition prevents exocytosis of IL-1β [78]. As previously mentioned (section 1.2.3), HMGB1 and IL-1β release is induced by inflammasome activation. The discovery of inflammasomes and how they are regulated was not well known at the time of the LPC study and the activation of effector caspases was thus not investigated [77]. NLRP3

inflammasome activation is dependent on potassium efflux and LPC is known to induce such an event [79, 80]. It is thus likely that studies involving LPC as an inducer for HMGB1 release actually mediate via inflammasome activation. Activation of several inflammasomes is known to induce release of HMGB1 [17, 18] and to date hyperacetylation of HMGB1 has only been described for the NLRP3 [18] and NLRC4 inflammasome (**PAPER II**).

2.3 Inflammatory functions

The extracellular effects of HMGB1 are diverse and include the induction of other inflammatory mediators, migration, differentiation and proliferation. Necrotic cells induce an inflammatory response whereas necrotic HMGB1 deficient cells do not, thus emphasizing the role of HMGB1 as an inducer of sterile inflammation [57]. The pleiotropic functions of HMGB1 are likely dependent on its capacity to activate diverse receptors. Several members of the TLR family including TLR2, TLR4 and TLR9 have been implicated in mediating HMGB1-induced inflammatory responses (**Figure 3**) [81, 82]. The ability of extracellular HMGB1 to induce cytokine production is at present mainly considered to be mediated via activation of the TLR4/MD2 complex and requires both MyD88 and TRIF signal transduction [83]. Conversely, the chemotactic effects of HMGB1 are induced via activation of the receptor for advanced glycation end-products (RAGE), but are also believed to be mediated in synergy with CXCL12 via CXR4 [84]. HMGB1 has also been shown to interact with CD24-SiglecG/10 that negatively regulates HMGB1 stimulatory activity [85]. The general view is that HMGB1-induced TLR activation and cytokine induction mainly occurs in myeloid cells whereas RAGE activation occurs in neutrophils, endothelial and neural cells.

Early reports on the endogenous effects of HMGB1 were widely questioned and attributed to the presence of contaminating TLR ligands such as LPS or bacterial DNA in the studied batches of recombinant, bacterially expressed HMGB1. This contested issue led to the discovery that HMGB1 could form complexes with and act in synergy with multiple ligands. Highly purified HMGB1 was for a long time believed, at least partly, to have little if any intrinsic cytokine-inducing effects, as reviewed in [86]. The ability of HMGB1 to interact with other inflammatory mediators, at least *in vitro*, can synergistically enhance the effect up to 1000-fold as compared to adding the stimuli alone [87-89]. This activation by such complexes or hetero-oligomers is mediated through the binding partner's cognitive receptor [88]. This synergistic effect has been reported for binding partners with both extracellular and intracellular receptors and includes IL-1α, IL-1β [89], LPS [90], CpG-DNA [82] and PAM_3CSK_4 [88]. HMGB1 thus acts as an innate immune sensor that can enhance the effect of other immune-stimulatory molecules. Such interactions and their importance *in vivo* remain to be elucidated. This controversy subsided with the discovery of the importance of the HMGB1 redox status for the activity of HMGB1 (**PAPER I**)[91, 92].

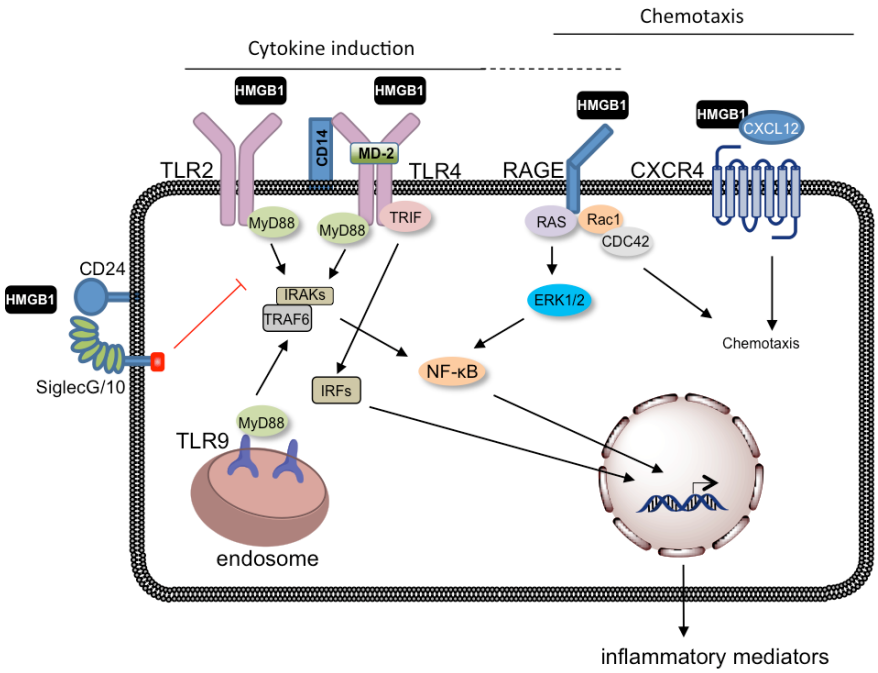

Figure 3: HMGB1 induced PRR activation*. A schematic overview of proposedl HMGB1 receptors and intracellular signal transduction. The diverse effects of extracellular HMGB1 are regulated by the capacity to interact and activate distinct PRRs. The cytokine-inducing effects are mainly mediated via TLR4 activation that requires both MyD88 and TRIF signal transduction and consequently leads to the production of pro-inflammatory mediators. The migration-inducing effects are mediated via RAGE or via CXCR4 when in a hetero-complex with CXCL12.*

2.3.1 HMGB1 and TLR4

Increasing evidence indicates that the intrinsic cytokine-like properties of HMGB1 are mainly mediated via TLR4. The principal and most studied activator of TLR4 is the prototypic PAMP molecule LPS (or endotoxin), a vital structural component of the gram-negative bacterial cell wall. In addition to HMGB1, other DAMPs have been suggested to signal through TLR4 but have also suffered from the skepticism that endotoxin contamination may be responsible for the suggested interaction [93].

Optimal signaling through TLR4 requires the co-receptors MD2 and CD14 and recognition of LPS is dependent on MD2 whereas CD14 confers sensitivity. Upon activation TLR4 oligomerizes and the C-terminal intracellular TIR domain recruits downstream adaptor and signaling molecules. The interaction between HMGB1 and TLR4 was first suggested by Park et al. in 2004 [94]. We have recently demonstrated the importance of MD2 in HMGB1 recognition and TLR4 activation [95].

The structure-to-function regulation of proteins by cysteine redox changes is well established [96]. The fact that cysteines are one of the least abundant amino acid in proteins but one of most frequently conserved suggest that they are highly important in protein function [97]. The biochemical properties of the cysteine thiol side-chain makes it highly reactive and the thiol may be oxidized, depending on the surrounding environment, in increasing oxidative order to cystine (disulfide), sulphenic (SOH), sulfinic (SO_2H) or irreversibly to sulphonic acid (SO_3H). Methionine side chains also contain a sulphur atom but are

less susceptible to oxidation than cysteines [98]. The regulation of HMGB1 function by cysteine redox changes has gained a lot of attention during the recent years [99]. HMGB1 contains three highly conserved cysteines in positions C23, C45 and C106. C106 is oxidized following non-immunogenic apoptotic cell death as a result of concomitant and excessive reactive oxygen species (ROS) production and regulates immune tolerance [100]. The direct cytokine-inducing effect of HMGB1 via TLR4 activation was not generally accepted, however, until the importance of cysteine redox was revealed. The cytokine-inducing effects of HMGB1 were first defined by peptide-mapping and this effect resided within a 20 amino acid C106-spanning peptide (89-108). Furthermore, a C106 thiol was required for the binding of HMGB1 to TLR4/MD2 and for activation of cytokine-induction in macrophages [91]. This clearly concords with the fact that HMGB1 in apoptotic cells contains an oxidized C106 induces immune tolerance [100]. The overall reductive intracellular environment normally keeps protein cysteines in a reduced thiol state. However, the more oxidative extracellular environment or excessive intracellular oxidative stress promotes the formation of a disulfide-bridge between the two cysteines in box A (C23-C45) and improves protein stability [101]. However, there are always two sides of a coin. ROS are normally scavenged by antioxidant systems composed of soluble antioxidants and antioxidant enzymes. The antioxidant protein thioredoxin (TRX) has been proposed to interact with HMGB1 and to maintain its cysteines as thiols within cells [101]. The presence of this disulfide bridge is equally important as C106 in its thiol form to confer HMGB1-mediated cytokine induction and this suggests that cysteine redox acts as a functional switch for HMGB1 inflammatory effects (**PAPER I**)[92, 102, 103].

2.3.2 HMGB1 and other receptors

Many other receptors besides TLR4 have also been described to mediate the extracellular functions of HMGB1. The first and perhaps one of the most described receptors is RAGE [104, 105]. RAGE is a member of immunoglobulin superfamily and its principal activators are advanced glycation end-products (AGEs). RAGE and AGEs are elevated and have a pathogenic role in several diseases [106, 107]. AGEs are non-enzymatically modified glycoproteins and are elevated in many inflammatory conditions. RAGE can also bind other ligands including members of the S100 family [108], amyloid-β-protein [109] and phosphatidylserine [110]. Many studies have demonstrated that RAGE is required for HMGB1-induced chemotaxis for many cell types including dendritic cells and neutrophils [105, 111, 112]. The interaction between RAGE and HMGB1 also has redox-dependent requirements, but in contrast to TLR4 activation all three cysteines must be in a reduced thiol form in order to induce migration via this receptor. The ability of HMGB1 to induce migration via RAGE is likely cell-type specific and some cells require the expression of CXCL12 induced by an HMGB1-activated RAGE receptor. In that case HMGB1 synergistically enhances the chemotactic effects of CXCL12-induced CXCR4 receptor activation, thus suggesting an indirect effect of RAGE in migration induced by HMGB1 [84, 102]. Other receptors have also been suggested to mediate extracellular effects of HMGB1, as reviewed in [67].

2.4 HMGB1 in disease

HMGB1 has been proposed as a significant contributor to pathogenesis in a plethora of diverse diseases in a multitude of publications. A common denominator for the diseases is the feature and importance of inflammation in their pathogeneses. HMGB1 is known to contribute to disease pathogenesis in both infectious diseases and non-infectious diseases caused by sterile inflammation , as reviewed in [113]. HMGB1 can be considered as a late inflammatory mediator in infectious diseases in contrast to sterile inflammatory diseases in which HMGB1 acts as an early mediator of inflammation.

2.4.1 Sepsis

Sepsis is a major clinical problem with a high mortality rate and is often caused by systemic gram-negative bacterial infection. The host-response during severe sepsis and septic shock resulting from systemic exposure to bacterial components becomes aberrant and excessive and causes the release of early pro-inflammatory mediators such as TNF and IL-1β. These cytokines peak within 1-2 hours and their increased systemic levels return to baseline after a couple of hours, resulting in a narrow window for therapeutic intervention. In contrast to other cytokines, HMGB1 is released in a delayed fashion and serum HMGB1 levels peak at 16-32h after induction of experimental sepsis. Septic patients have increased serum levels and the highest levels of HMGB1 were recorded in non-survivors. Furthermore, systemic injection of HMGB1 in mice results in characteristics similar to those of severe sepsis and is lethal [66]. In addition, post-septic patients suffer from cognitive impairment and the extent of this impairment correlates with HMGB1 levels [114]. Targeting HMGB1 with specific antibodies or inhibiting the release of HMGB1 ameliorates experimental sepsis and increases survival in septic mice [17, 66]. Antagonizing HMGB1 at delayed time points, as late as 24h after the sepsis-causing insult, still has profound beneficial effects in sepsis models [66]. This is in contrast to therapies targeting the early cytokines (e.g. TNF, IL-1β, MIF), which have little or no effect on disease outcome when administered at later time points [115-117]. Infectious diseases and especially sepsis likely serve as prototypical models in which HMGB1 acts as a late inflammatory mediator.

2.4.2 Ischemia reperfusion (I/R) injury

The restriction of tissue blood flow causes a hypoxic state in a tissue and when blood flow returns and reperfuses the tissue, a sterile inflammatory response is initiated. These events may occur in many types of organs as a result of trauma, transplantation or stroke [118]. I/R events induce necrotic cell death but also the change in physiological condition can stress cells to release DAMPs, including HMGB1. In an experimental model of liver I/R, HMGB1 is already detected in the serum of mice after 1h. Monoclonal anti-HMGB1 antibody treatment significantly decreased liver injury whereas administration of HMGB1 worsened liver I/R. Interestingly, TLR4-defecient mice have significantly less liver injury than do wild type mice after induction of liver I/R [119], indicating an importance of HMGB1-induced TLR4 activation in I/R pathogenesis. Beneficial effects of HMGB1-blockade have been described in other cases of organ I/R injuries as well, including both in brain and kidney [72, 120].

2.4.3 Acetaminophen drug induced liver injury

HMGB1 is involved in the pathogenesis of several liver diseases and models thereof [121]. The potentially hepatotoxic compound acetaminophen (APAP) is one of the most widely used over-the-counter anti-pyretic and analgesic drugs. APAP overdoses are the predominant cause of acute liver failure in the western world. Intentional (suicide attempts) or unintentional APAP-overdoses constitute a substantial healthcare burden and account for approximately 26000 hospitalizations and 500 deaths a year in the US alone [122]. APAP-induced liver injury is widely used as a model to study chemically induced hepatotoxicity. Although generally safe at therapeutic doses, APAP has a narrower window of safety as compared to equivalent drugs such as non-steroidal anti-inflammatory drugs (NSAIDs). The conventional treatment (N-acetyl cysteine) for APAP intoxication has limited therapeutic effect if not administered within the first 8h post-APAP ingestion [123].

Hepatocytic cell death as a result of an APAP overdose induces the release of several DAMPs as expected, including HMGB1 [124, 125]. Targeting HMGB1 with polyclonal antibodies (pAbs) has profound effects on hepatotoxicity and immune cell infiltration [124]. Interestingly, the measurement of serum HMGB1 from APAP-overdose patients is a significantly better prognostic biomarker for disease outcome as compared to measurement of the commonly used liver injury marker alanine amino transferase (ALT). Hyperacetylated HMGB1 is even better than total HMGB1 as a biomarker, suggesting an involvement of activated innate immune cells in the pathogenesis of APAP intoxication [126, 127]. In addition, this clearly indicates that measuring specific PTMs may be beneficial when investigating the significance of HMGB1 as a biomarker. Both RAGE and TLR4 have been implicated as receptors mediating the pathogenic effects of HMGB1 in this disease [128, 129].

2.4.4 Chronic inflammatory autoimmune diseases

Several studies have identified HMGB1 as a mediator of chronic inflammatory autoimmune diseases. Rheumatoid Arthritis (RA) is the most common rheumatic disorder with a prevalence of around 1%, has unknown etiology and is characterized by chronic inflammation and joint swelling in one or more joints. Furthermore, patients with RA have decreased life expectancy, a reduced quality of life and this patient group represents a significant societal economic burden. The pathogenic role of HMGB1 in chronic arthritis is evident. Several key points emphasize the pathogenic contribution of HMGB1 to arthritis: 1) HMGB1 is highly expressed in the synovial membrane and synovial fluid of arthritis patients [130, 131]; 2) targeting HMGB1 release or function reduces severity and tissue destruction in experimental arthritis [132, 133]; 3) intra-articular injection of recombinant HMGB1 induces severe arthritis [134]. Drugs used to symptomatically treat arthritis also have the capacity to reduce the release of HMGB1 from human monocytes [68]. The etiology of arthritis is complex and as with acute sterile inflammation, HMGB1 likely induces and perpetuates inflammation but now in a chronic setting. Although chronic inflammation is difficult to define in an exact manner, it is regarded as an inflammation with a prolonged duration whereby inflammation, tissue destruction and attempts to repair occur simultaneously. In chronic inflammatory

diseases (**PAPER III**) and in contrast to acute inflammatory conditions [124], diverse HMGB1 cysteine redox isoforms have been recorded, suggesting that HMGB1 may be functionally diverse in different inflammatory settings.

Chronic arthritis is not only restricted to adult individuals. Juvenile idiopathic arthritis (JIA) is, likewise to RA, a heterogenic chronic inflammatory disorder and the most common cause of arthritis in children. The worldwide prevalence of JIA is considered to range between 0.1-0.4% [135] and is manifested by articular and extra-articular chronic inflammation that could potentially cause joint destruction and disabilities. According to the International League of Association for Rheumatology (ILAR), JIA is divided into 7 sub-diagnoses [136]. The common denominators for all sub-diagnoses are arthritis for at least 6 weeks, disease onset before the age of 16 and the exclusion of other differential diagnoses [135]. The most common sub-diagnosis is oligoarticular arthritis (~40-50%) followed by polyarticular arthritis (~30%). Polyarthritis is divided in Rheumatoid Factor (RF)-negative or RF-positive polyarthritis of which the latter is of erosive nature and shares immunological and clinical features with RA. In contrast to RA, some JIA patients have few or no symptoms as adults. JIA patients with a polyarticular (mainly RF-positive) diagnosis are least likely to go into remission [137, 138]. Like other autoimmune diseases, JIA is multifactorial and risk factors for development of JIA are both environmental and genetic. HMGB1 has recently been shown to be highly expressed in the synovial fluid of JIA patients as compared to in plasma, and synovial fluid HMGB1 levels correlated with an early onset of disease but not with inflammatory markers [139]. No specific experimental JIA model exists and experimental *in vivo* studies are restricted to available arthritis models.

2.4.5 HMGB1 and tissue regeneration

The extracellular effects of HMGB1 and other DAMPs are not only detrimental. HMGB1 and many other DAMPs have the additional capacity to induce tissue regeneration. Cell migration can be of pathological nature but it is also an important physiological event in the dynamic process of tissue regeneration following injury. HMGB1 induces migration of non-innate immune cells such as keratinocytes, mesioangioblasts [140], smooth muscle cells [141], endothelial cells, hepatic stellate cells [142] and fibroblasts [143], in addition to migration of immune cells [144]. In general, the requirement of RAGE in HMGB1-induced migration is evident. However, concomitant activation of CXCR4 by HMGB1-CXCL12 heterodimers also likely contributes to increased migratory features during tissue regeneration [84, 102].

As previously mentioned, HMGB1 can be released as a result of hypoxia both *in vitro* and *in vivo* [71]. The induction of migration in combination with stimulation of angiogenic factors highlights the importance of HMGB1 signaling in ischemic tissue for neovascularization [145]. TLR4 has also been suggested to be selectively required for HMGB1-mediated corneal neovascularization with a moderate role of RAGE [146]. Nonetheless, the regenerating capacity of HMGB1 and its receptor dependence is likely tissue and cell type-specific. The angiogenic effects of HMGB1 can promote cancer tumor growth [145]. Rapidly growing tumors generate hypoxic and necrotic areas within the tumors with an increased expression of angiogenic and pro-

tumorogenic factors and induce migration of tumor-associated macrophages [147]. The pro-tumorogenic effects of HMGB1 can be ameliorated by neutralizing antibody treatment [148].

2.5 Opposing HMGB1 inflammatory effects

Interfering with HMGB1 inflammatory function is beneficial in many experimental disease models. Therapeutic targeting of HMGB1 can be accomplished by either targeting its expression and release or antagonizing and neutralizing the extracellular effects, as reviewed in [149].

Targeting HMGB1 release may be achieved by antagonizing the inflammatory mediators that induce HMGB1 release, and thus indirectly affect HMGB1 activities. Successful inhibition of the nuclear-to-cytoplasmic HMGB1 translocation and release reduces extracellular levels of HMGB1 in many disease models [67]. The anti-malaria drug chloroquine reduces HMGB1 release in LPS/IFNy-stimulated macrophages *in vitro* [68] and oxaliplatin *in vivo* [150]. Recent reports have demonstrated that pre-treatment with chloroquine significantly reduces HMGB1-induced serum levels in models of liver I/R and sepsis [151, 152]. In the liver I/R model, chloroquine treatment inhibited liver injury at early time points but had opposite effect at later time points [151]. This indicates a dual role of chloroquine in liver I/R and suggests that HMGB1 targeting therapies are unfavorable in later phases of inflammation when tissue regeneration occurs. Interestingly, in APAP-induced liver injury functional HMGB1 redox isoforms are present in different stages of inflammation with the more oxidized HMGB1 isoform present during the resolving phase of inflammation (**PAPER I**). This isoform has been proposed to be tolerogenic [100] and may have a role as modulator of the resolving inflammatory phase and induction of tissue repair. Taken together, therapeutic targeting of HMGB1 may be beneficial in some diseases at specific stages but it may also be detrimental in others. Long-term investigation of anti-HMGB1 treatment and the potential adverse effects of such therapies need to be further established.

Targeting extracellular HMGB1 may be achieved by using specific antibodies, receptor blocking agents or soluble receptors. The use of HMGB1-specific polyclonal antibodies (pAbs) has been a very successful strategy in experimental models. Although pAbs have aided in understanding the pathogenic role of HMGB1 in many diseases, they possess little therapeutic value in human diseases. Only a few monoclonal antibodies (mAbs) have demonstrated equivalent therapeutic effects to pAbs *in vivo* [149]. In contrast to pAbs, mAbs could potentially be of therapeutic value in human disease. Extracellular HMGB1-induced effects can also be antagonized by administration of the box A domain [67]. The box A domain is believed to act as a receptor antagonist inhibiting HMGB1 function. The *in vivo* presence or significance of such truncated HMGB1 variants is unknown. However, in comparison to antibodies, the serum half-life of box A or smaller peptides/molecules targeting HMGB1 function would be significantly shorter and, in addition, such therapies could potentially pass cellular membranes and induce undesirable effects.

Several mechanisms negatively regulate HMGB1 extracellular effects. HMGB1 binds to CD24 and CD24-Siglec10/G activation reduces HMGB1-induced NF-κB signaling, thus providing a negative feedback

mechanism. CD24-deficient mice demonstrate increased susceptibility to HMGB1- and heat shock protein (HSP)-stimulation, but not to PAMP activation. These findings indicate that CD24 discriminates between DAMPs and PAMPs and acts as a modulator of DAMP activation [85]. Other receptors such as T cell immunoglobulin mucin-3 (TIM-3) also bind and negatively regulate HMGB1 function [153]. Some proteins have the capacity to cleave and degrade HMGB1 and hence modulate inflammatory function. Thrombomodulin cleaves the peptide bond between the amino acid residues R10-G11, and CD26 cleaves the peptide bond between P9-R10 [154, 155]. While both of these N-terminally cleaved HMGB1 variants display reduced extracellular effects *in vitro,* any *in vivo* significance of such HMGB1 variants is currently unknown.

3 AIMS

When this thesis work was initiated the pathogenic contribution of HMGB1 in multiple diseases was well established. The extracellular actions of HMGB1 and especially the cytokine-inducing effects had for a long time been a matter of debate. Recent findings at that time suggested that cysteine redox isoforms could greatly impact the extracellular functions of HMGB1. The presence of such cysteine redox isoforms *in vivo*, especially in chronic inflammation, was ill defined. Additionally, although accumulating experimental data suggested that HMGB1 neutralization might be a new avenue for anti-inflammatory therapy, additional work was needed to define drug candidates that could be forwarded to clinical trials.

The aim of my thesis work was to evaluate functional regulation of HMGB1 *in vivo* and *in vitro* and to study active release mechanisms. Furthermore, I set out to define the potential therapeutic potential of an anti-HMGB1 mAb (2G7) as a new anti-rheumatic treatment and to progress such therapy further towards treatment of human diseases.

More specific aims of this thesis were to:

1. Investigate the regulation of HMGB1 by modulation of cysteine redox states (**PAPER I**).

2. Explore a mechanism of active HMGB1 release, without priming signals, and whether metabolic changes as a result of TLR agonism alter the redox isoforms of HMGB1 (**PAPER II**).

3. Explore the functional contribution of HMGB1 to the pathogenesis of JIA, release mechanisms activated and cellular sources of the released HMGB1, by defining HMGB1-PTMs present (**PAPER III**)

4. Evaluate the therapeutic, anti-rheumatic potential of an HMGB1-specific monoclonal antibody in experimental models of arthritis (**PAPER IV**).

5. Develop a potential therapeutic chimeric anti-HMGB1 antibody for future clinical trials (**PAPER V**).

4 METHODOLOGICAL CONSIDERATIONS

In this section I present a brief overview of and discuss selected methods used in the studies in this thesis. For more comprehensive details, I refer to the respective papers.

4.1 Recombinant protein production

In order to study the cytokine-inducing effects of HMGB1 in a controlled manner we used bacterially expressed recombinant proteins. The conservative amino acid sequence between mammals (99% sequence homology) clearly indicates functional trans-species homology. Throughout most of these studies I have used *Escherichia coli (E. coli)* expressed calmodulin-binding protein (CBP)-tagged rat HMGB1. Rat and mouse HMGB1 display 100% amino acid homology and only two amino acids differ from human HMGB1. The amino acid shifts are located within the C-terminal tail where a glutamic acid is substituted with an aspartic acid and *vice versa* (D189E and E202D). The similar properties of these amino acids are not likely to affect the protein function and experiments using human HMGB1 demonstrate equal properties (data not included). The CBP-tag itself does not affect HMGB1 function [156]. Overexpressed HMGB1 was DNase treated due to the tendency of HMGB1 to bind bacterial DNA and LPS removal was performed using Triton-X114 purification. Endotoxin contamination for all batches used was measured using limulus amoebocytic assay, was below the detection limit (<3 endotoxin units/milligram protein) and thus, was considered as endotoxin- and DNA-free. The presence of bacterial components in recombinant preparations of HMGB1 was likely equally responsible as redox differences for the unambiguous conception with HMGB1 ability to induce cytokines over the past decades [86]. The lack of post-translational machinery in bacteria thus makes redox the most likely modification affecting its function.

4.2 Experimental animal models

4.2.1 Arthritis models

Numerous different experimental arthritis models have been developed in different species and are either induced or of spontaneous nature. Although all models share partial features with RA, none truly reflect all features of RA. The common denominator for most arthritis models is that they are normally studied in their acute inflammatory phase and are not allowed to reach their chronic inflammatory phase for ethical reasons. Evaluation of RA-modulating therapeutics would perhaps be better investigated in a chronic phase of the disease. Most treatments for RA are currently used due to serendipitous observations. In contrast, the treatment with anti-TNF biologics was the first rationally observation-based experimental treatment developed and has progressed the use of biological therapies used in RA today. The effect of disease modifying drugs in experimental arthritis is highly strain- and species-specific [157, 158]. HMGB1 has been implicated to mediate pathogenesis in the models described below and these were thus the obvious choice for studying the effect of a mAb targeting HMGB1 in this thesis (**PAPER IV and IV**) [132, 159].

4.2.1.1 Collagen-induced arthritis (CIA)

In this model arthritis is induced by a subcutaneous injection of heterologous bovine collagen type II (CII) in Freund's complete adjuvant at the base of the tail of mice (DBA/1) followed by a boost with CII in incomplete Freund's adjuvant at day 21. This causes a breaking of immune tolerance, induces formation of pathogenic anti-collagen antibodies, directs an immune activation to the joints and induces polyarthritis with severe cartilage and bone erosion. Although the CIA model shares several features with RA, it is mainly studied during its aggressive acute phase that only partially reflects RA pathogenesis. Nonetheless it is considered to be the *in vivo* experimental golden standard for RA. Other models may better reflect human RA and the course of chronic joint inflammation. Nevertheless, the major histocompatibility complex (MHC) class II-associated susceptibility together with the dependence of T-cell regulated responses in CIA are shared with RA [160].

4.2.1.2 Spontaneous chronic polyarthritis

DNase II deficient mice die as embryos due to their inability to digest chromosomal DNA and the ensuing excessive IFN-β production. Mice also deficient in IFN type I receptor (DNaseII$^{-/-}$xIFN-IR$^{-/-}$) are born normal but develop chronic polyarthritis starting around 8 weeks of age. These mice share several features with human RA including joint swelling, histopathological features and increased levels of TNF, matrix metalloproteinase 3 (MMP-3), RF and anti–citrullinated protein (anti-CCP) antibodies. In contrast to human RA and to the CIA model the progression of arthritis in these mice does not involve a T-cell response and is most likely dependent on macrophages activated by frustrated phagocytosis caused by the overwhelming quantity of undigested DNA [161].

4.2.2 APAP-induced liver injury

APAP is metabolized in the liver by hepatocytes with a small fraction (~5%) bio-activated by the cytochrome P450 (CYP450) system, generating the highly reactive intermediate metabolite N-acetyl-para-benzoquinone imine (NAPQI) metabolite [11]. NAPQI is normally detoxified by the intracellular antioxidant glutathione (GSH) and excreted via urine. However, when formation of the NAPQI exceeds the capacity of the intracellular hepatic GSH pool, NAPQI covalently reacts with protein sulfhydryl groups (i.e. cysteines) [12]. The formation of NAPQI-protein conjugates results in loss of protein function, extensive cellular stress, hepatocyte cell-death and ultimately HMGB1 release. The extent of these conjugates correlates with the extent of liver injury [162]. Existing therapeutic strategies for APAP overdose used today are based on mechanistic studies in experimental models and target the initial toxic NAPQI metabolite that is formed during APAP bio-transformation in hepatocytes [163]. Generally, in order to achieve significant hepatotoxicity the mice are fasted to reduce levels of GSH and ATP and thus promoting inflammatory cell death rather than apoptotic cell death [124]. This generates massive release of HMGB1 that exacerbates the post-injury inflammation and antibodies targeting HMGB1 significantly ameliorate disease pathogenesis [57, 124, 126, 129, 164].

The use of mice as the preferred rodent for investigating APAP toxicity was based on the fact that rats are generally less susceptible [165]. This is believed not to be an effect of a general difference of APAP metabolism but rather the associated mitochondrial dysfunction that is critical for the development of hepatocellular necrosis and is similar between mouse and human [166]. APAP-induced liver toxicity in mice thus constitutes an experimental model well suited for studies of HMGB1-mediated inflammation as well as allowing rapid screening and evaluation of HMGB1-neutralising therapies. APAP-induced liver toxicity in mice was used in **PAPER V** to evaluate the effects of a humanized, chimeric anti-HMGB1 antibody.

4.3 HMGB1 and clinical samples

The collection of synovial fluid from JIA patients was carefully considered to ensure that HMGB1 was not a result of general cellular necrosis as has been previously been reported [167](Erik Sundberg, unpublished data). As shown in **PAPER III**, the levels of HMGB1 did not correlate with lactate dehydrogenase (LDH) activity. The gender distribution of the randomly selected patients in **PAPER III** generated a diverse female-to-male ratio as compared to the 2:1 ratio apparent in the clinic. This effect was most likely caused by the small sample size since our bio-bank encompasses synovial fluid samples with a clinically comparable gender ratio. However, a partial biased selection of patients may have occurred since only patients with >150µl synovial fluid were selected for LC-MS/MS analysis, and this needs to be further validated. These patients also received various medications that could possibly interfere with HMGB1 function or release [68, 168].

HMGB1 has been implicated in the pathogenesis of a multitude of diseases and thus mandates reliable assays for quantification in biological samples. Serious concerns regarding detection assay validity arise from the fact that HMGB1 clearly exists in several isoforms and has the ability to bind to other molecules. It was previously published that certain antibodies are only able to detect HMGB1 in its reduced condition and that oxidized HMGB1 remained undetectable by immune blotting [169]. The suitability and selectivity of different assays and the antibodies used for detection towards different HMGB1-isoforms should hence be taken into consideration. The formation of complexes with exogenous or endogenous partner molecules in addition to immune complexes could further complicate analysis and generate underestimated absolute values [170]. Throughout this thesis I have used antibodies that do not have a preference for certain isoforms of HMGB1.

A concern that might be raised is the potential that the commonly used ELISA might be affected by the presence of HMGB1 autoantibodies. The presence of such autoantibodies is not a concern in most *in vitro* samples but may be importantly noted in samples from RA or systemic lupus erythematosus (SLE) patients with a recorded history of HMGB1 specific autoantibodies. The presence of such autoantibodies in JIA patients is still unknown, but unpublished data from samples in our bio bank indicates a low frequency of <2% (Hanna Schierbeck, personal communication).

4.3.1 Characterization of HMGB1 PTMs

The importance of HMGB1-specific PTMs is critical in order to define functional properties and release mechanisms. Isolation of HMGB1 for liquid-chromatography tandem mass-spectrometry (LC-MS/MS) analysis (used in **PAPER I**, **II** and **III**) was performed by immunoprecipitation (IP) with a non-isoform-specific antibody [171]. The IP step was performed to reduce the background noise of other synovial fluid proteins and to improve sensitivity and resolution for HMGB1-specific peptides. The use of LC-MS/MS allows a sensitive characterization of targeted PTMs with reliable *de novo* sequencing derived from the fragmentation of peptides in the second round of MS. During *de novo* sequencing the peptide is fragmented along the peptide backbone and generates an MS/MS spectrum (mainly b and y ions, due to the fragmentation method collision-induced dissociation (CID)). There are also possible labeling approaches developed for MS/MS, including spike-in synthetic peptide standards that can be utilized for reliable quantitative studies. However, we did not utilize a labeling approach but the nature of mass-spectrometry still allows us to compare extracted area under the curve (AUC) values for specific peptides between samples in a qualitative but not in an absolute quantitative manner. The transient states cysteine redox was addressed by labeling cysteine thiols with alkylating agents. In order to separate cysteines from disulfide-forming cysteines, I utilized a two-step alkylating protocol with a reducing dithiothreitol (DTT) step in between. The alkylating agents were of different molecular weights and added in excess which facilitated identification of the redox state of HMGB1 (e.g. iodoacetamide, *N*-ethylmalemide (NEM) or d5-NEM). Isolated and alkylated HMGB1 was subsequently digested by the protease Glu-C (cleaves at the C-terminus of glutamic acids, utilized for analysis of acetylation) or trypsin (cleaves at the C-terminus of arginine or lysine, utilized for analysis of redox). This provides an accurate qualitative measurement of HMGB1 PTMs. However, a high throughput antibody based assay would be preferable for analysis of all isoform-specific HMGB1 variants in biological samples, but no such assay is currently available.

5 RESULTS AND DISCUSSION

5.1 Redox regulation of HMGB1 extracellular function

The proposition that HMGB1 possesses intrinsic cytokine-inducing capacity was initially met by significant resistance in the field, and rightly so, since several conflicting reports were concurrently published [86]. The initial evidence supporting that PTMs, and more specifically cysteine redox status, could modulate HMGB1 function was described in apoptotic cells that are known to promote immune tolerance, in contrast to necrotic cells. HMGB1-deficient apoptotic cells failed to induce immune tolerance and blockade of caspase-3/7-dependent ROS production prevented the induction of immune tolerance by apoptotic wild-type cells [100]. It was further demonstrated that the immune tolerance was dependent on C106 of HMGB1 being oxidized. C106 has in addition been demonstrated to be key for HMGB1-mediated cytokine induction [91, 100]. HMGB1 contains three highly conserved and redox susceptible cysteines. The initial report by Kazama et al. suggested that redox properties of C23 and C45 are redundant with respect to immune tolerance [100]. However, C23 and C45 readily form a disulfide bridge that improves protein stability and structural changes associated with such a modification could greatly impact protein functions [101, 172]. The increased understanding of the impact of redox modifications for HMGB1 protein function also clarified the problems with batch-to-batch variations of recombinant HMGB1. The use of different purification techniques impacts protein redox status and thereby its function. Furthermore, commercially available recombinant proteins are normally purified in the presence of a reducing agent, usually DTT, and such proteins fail in induction of cytokines, at least in our experience. Hence the ambiguities of cysteine redox and its impact on HMGB1 inflammatory function led us to hypothesize that redox changes were the most likely PTM affecting HMGB1 cytokine-inducing function, leading to the studies presented in **PAPER I**.

LC-MS/MS analysis revealed an astonishing correlation between the defined redox states of all three cysteines in numerous batches of recombinant HMGB1 tested and their ability to induce cytokine induction. In **PAPER I** we could demonstrate that C23 and C45 form a disulfide bridge in recombinant HMGB1 with the capacity to induce cytokines. We could also confirm the previous known functional importance of a non-oxidized C106 for the cytokine-inducing function [91]. The proposed redundancy of C23 and C45 in immune cell regulation may perhaps be explained by different experimental setups as the original study investigated induction of immune tolerance by apoptotic cells and not a direct ability of HMGB1 to induce cytokine-production [100]. Our own and other data clearly support the notion that a disulfide bridge is required between C23 and C45 with a simultaneous C106 thiol side chain for HMGB1 to have cytokine-inducing properties [92, 102]. Other cysteine oxidative modifications [173], besides the ones mentioned in this thesis, could potentially elicit similar or additional functional effects and should be considered in future studies, especially from an *in vivo* point of view. Thus further characterization of individual modifications present on HMGB1 recovered from biological samples might help in understanding the diverse biological effects of HMGB1 *in vivo*.

When we oxidized cysteine side chains to sulphonyls through hydrogen peroxide treatment we clearly induced a functional transition of HMGB1 to a non-cytokine inducing isoform. In addition, exposure to DTT clearly reduced the C23-C45 disulfide bridge and demonstrated a similar functional transition as with oxidative treatment. Regulatory effects of cysteine redox changes can likewise be detected in human and mouse macrophages/monocytes, supporting trans-species similarities. Such cysteine-dependent regulation of HMGB1 activity was further confirmed *in vivo* in the study by Venereau et al. and thus gave supporting evidence that the effect of cysteine redox modulation is not an *in vitro* artifact [102]. Hence, intra- or extra-cellular environmental redox changes can regulate the functions of HMGB1 and dictate its cytokine-inducing effects. Changes in the overall redox state both in the intra- and extra-cellular compartments *in vivo* are evident during different inflammatory stages and alter the inflammatory properties of HMGB1 (**PAPER I**)[174]. Extracellular activities of other DAMPs can also be modulated by redox changes. For example, the extracellular function of certain S100 protein family members are regulated by cysteine redox modifications [175]. Taken together, our results presented in **PAPER I** together with other studies suggest that overall DAMP function can be modulated by changes in the redox environment and thus impact on the evolving inflammatory response.

The studies in **PAPER I** were performed as a direct follow-up study of the findings by Yang et al. wherein binding studies had demonstrated an affinity of HMGB1 to the TLR4/MD-2 complex, and that such an effect could be abrogated by chemical modulation or site-directed mutagenesis of C106 [91]. Modulation of HMGB1 cysteine redox status could only partly suppress the cytokine induction. This might be explained by the fact that the proposed 'cytokine-inactive' variants are weak TLR4 agonists, thus having antagonistic properties but still inducing a weak cytokine production *per se*. A similar reasoning is proposed for the TLR4 antagonistic effects evident with 'inactive' LPS derived from *Rhodobacter sphaeroides* (LPS-RS). Nonetheless, the potential suppressive effects of different HMGB1 cysteine isoforms on TLR4 activation by receptor ligation remain to be elucidated. A final consideration is that the highly reactive and transient state of cysteine side chains, except the sulphonic and in part sulfinic acid form, may revert to its cytokine-inducing state after addition to cell culture media.

The focus of **PAPER I** was the interaction of HMGB1 with the TLR4 complex, and the impact of other putative HMGB1 receptors on cytokine induction was not addressed. Such putative HMGB1 receptors, both intra- and extra-cellular, may of course influence the cellular responses to HMGB1 and might explain the partial cytokine reduction recorded with the all-thiol and sulfonyl-HMGB1 cysteine redox isoforms. Some studies have proposed the involvement of RAGE in HMGB1-mediated cytokine-induction. Although there is more compelling evidence supporting RAGE in the chemotactic effects of HMGB1 it is still possible that RAGE mediates and impacts on both migration- and cytokine-induction by HMGB1 either directly or indirectly. The HMGB1 redox requirements, or other PTMs for that matter, for cytokine induction via RAGE or additional HMGB1 receptors are still unclear.

The importance of the co-receptors CD14 and MD2 for HMGB1-induced TLR4 activation has recently been evaluated [83, 95]. This clearly indicates a similar recognition mechanism as for the bacterial-derived and principal TLR4 agonist LPS. It is also likely that CD14 is not required, but improves sensitivity to, HMGB1-induced TLR4 activation. Furthermore, there is a clear requirement of MD2 in HMGB1-mediated cytokine production both *in vitro* and *in vivo* [95]. Interestingly, there seems to be a discrepancy in LPS- and HMGB1-induced intracellular NF-κB signaling. In addition, proteolytic degradation of HMGB1 abolished NF-κB activity, further supporting the fact that HMGB1-induced responses are not due to endotoxin contamination [94].

Whether HMGB1 and LPS share the same binding site for TLR4/MD2 has been a matter of discussion. My colleagues and I have demonstrated that a tetramer peptide (FSSE) mimicking the sequence of the key TLR4/MD2-interacting HMGB1 region C106 (FCSE) bound to MD2 but not to TLR4 or HMGB1 [95]. This peptide could selectively reduce HMGB1-induced cytokine production *in vitro* and partly conferred protection in HMGB1-dependent liver injury models *in vivo*. It was thus suggested to provide a specific HMGB1-induced TLR4-activation blocking strategy. The weak TLR4 agonist LPS-RS antagonizes the inflammatory effects of HMGB1 both *in vivo* [146, 176] and dose-dependently *in vitro* **(Figure 4),** thus providing further support of the importance of HMGB1-TLR4/MD2 interaction for cytokine induction. Conversely, this might suggest that HMGB1 shares the same binding site to TLR4/MD2 as LPS, which is in contrast to the results of the FSSE peptide study. This discrepancy might be explained by several mechanisms besides receptor binding competition; steric or allosteric hindrance, down-regulation of TLR4 expression, induction of TLR4-negative feedback mechanisms and differences in biological half-life are all possible explanations. The fact that HMGB1 has the capacity to directly bind LPS and other DAMP/PAMP molecules [88, 90] provides another potential mechanism. In addition, preconditioning animals with non-stimulatory doses of HMGB1 was clearly protective in experimental models of sepsis [177] and liver I/R [178] suggesting that HMGB1 in low doses induce down-regulatory mechanisms of TLR4 signaling.

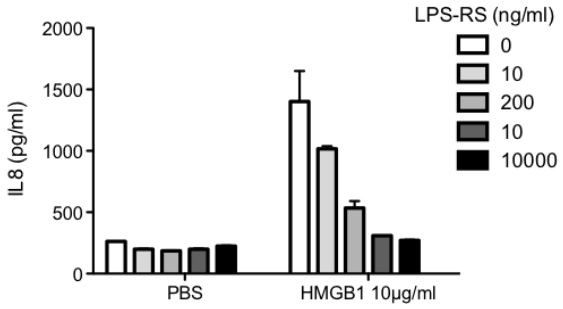

Figure 4: *TLR4 antagonism decreases HMGB1-induced production of IL8 in TLR4/MD2/CD14 expressing human embryonic kidney cells (HEK293) cells. Stimulation with the TLR4 activating HMGB1 redox isoform induced the production and release of IL8. TLR4 antagonism by the "inactive" LPS derived from Rhodobacter sphaeroides (LPS-RS) dose-dependently reduced the response to HMGB1 stimulation.*

5.2 Regulation of HMGB1 release and the functional connection

The regulation of HMGB1 release as a result of inflammatory stimuli is highly associated with hyperacetylation of lysine stretches within the NLS regions. It should be remembered that HMGB1 contains multiple lysines that are normally acetylated under non-stimulatory conditions [179]. It is the hyperacetylated form that is associated with an active release. Other PTMs have been suggested to regulate the cytoplasmic translocation but are likely accompanied by the acetylation of these key lysine residues. Several cell types likely possess the capacity to acetylate and translocate HMGB1 but only a few release significant quantities of HMGB1. One important observation is that inflammasome activation, specifically NLRP3 inflammasome, induces release of significant HMGB1 levels [17, 18]. Studies of how the regulation of the NLRP3 inflammasome modulates HMGB1 release are complicated by the fact that *in vitro* systems usually require a two-hit model.

The general consensus has been that the first hit (priming) induces acetylation and translocation of HMGB1, whereas the second signal causes assembly and activation of the multi-protein caspase-1 activating inflammasome machinery. However, whether inflammasome activation *in vitro* can regulate HMGB1 release without a priming signal was previously unknown. In **PAPER II** we utilized the fact that the NLRC4 inflammasome does not require a priming signal but is directly activated by the TLR5 agonist flagellin. In genetically engineered mouse macrophages, activation of the NLRC4 inflammasome resulted in the release of significant levels of HMGB1 without the requirement of a priming signal (**PAPER II**). Interestingly, NLRC4 inflammasome activation promoted acetylation of HMGB1 independently of priming. The released HMGB1 contained cysteine thiol side-chains, suggesting that HMGB1 was not released in its cytokine-inducing form. How the NLRC4 inflammasome induces HMGB1 acetylation remains unknown. Although difficult to elucidate due to the two-hit stimulation required, it is possible that the NLRP3 inflammasome also induces HMGB1 acetylation independent of priming. The priming signal for NLRP3 inflammasome activation is usually required *in vitro* but not necessarily *in vivo* [180], thus highlighting an important discrepancy when comparing *in vitro* and *in vivo* results.

Analysis of NLRP3 activation-induced release of HMGB1 suggests that it is partly the cytokine-inducing isoform that is released [18]. There is a requirement for ROS production for activation of the NLRP3 inflammasome [181]. Inhibition of ROS clearly reduces NF-κB activity and thus affects priming of NLRP3 [27, 182]. Furthermore, many stimuli that induce the release of HMGB1 are associated with ROS production and NF-κB activation. Triggers of the NLRP3 inflammasome, such as ATP and MSU, are also known to induce ROS production [181, 183]. In contrast, NRLC4 activation is independent of priming and ROS production although a priming signal is needed for the induction of pro-IL-1β (**PAPER II**).

We thus hypothesized that inflammasome activation alone does not induce the molecular transition of HMGB1 to its disulfide, cytokine-inducing form. Instead, the priming-induced ROS production is likely responsible for this regulatory mechanism. Indeed, priming of cells induced the transition of HMGB1 from its fully reduced form to the disulfide, cytokine-inducing isoform. ROS production as a result of innate

immune responses (mainly neutrophils and macrophages) to bacterial components was previously believed to be dependent on NADPH-oxidase (NOX) activation [184, 185]. However, during normal metabolism the bulk cellular ROS mainly originates from the mitochondria (mtROS) as byproducts of mitochondrial respiration [186]. New evidence has highlighted the importance of mtROS in the response to bacterial components activating cell surface, but not endosomal TLRs [187]. We thus speculated whether the functional transition of HMGB1 was dependent on mtROS or NOX-derived ROS. Blocking total ROS or mtROS inhibited the formation of the cytokine-inducing form of HMGB1 whereas pan-NOX inhibition did not (**Figure 5**). Importantly, inhibition of ROS did not affect the extent of NLRC4 inflammasome activation (data not included) or the quantity of HMGB1 release (**Figure 5**). Taken together, NLRC4 activation promotes the release of HMGB1 and the functional transition of HMGB1 during inflammasome priming is dependent of mtROS (**Figure 6**).

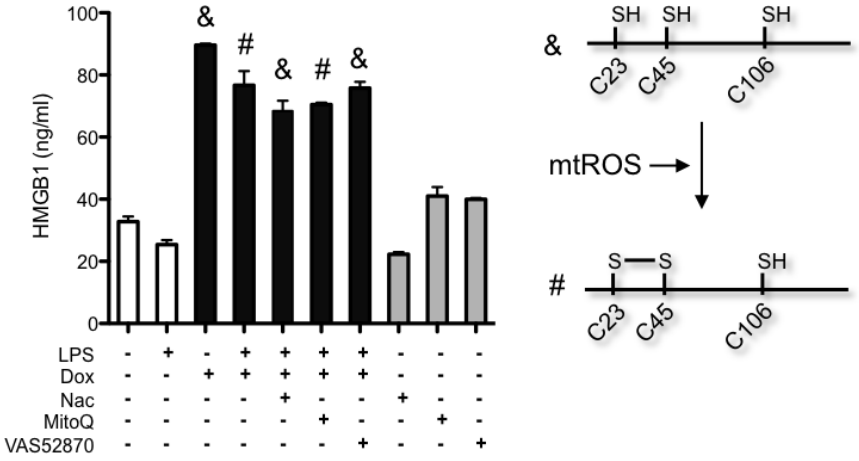

Figure 5: ROS production regulates the pro-inflammatory function but not the release of HMGB1*. Addition of doxycycline (Dox) induces the expression of the last 34 C-terminal amino acids of flagellin that subsequently activates the NLRC4/NAIP5 inflammasome, hyperacetylation of HMGB1 NLS regions and consequently HMGB1 release in its chemotactic reduced redox isoform (&). Addition of the classic priming signal LPS induced a functional transition of HMGB1 to its cytokine-inducing redox isoform (#). Blocking total cell ROS with N-acetyl cysteine (Nac) or mtROS production (mitoQ) did not inhibit the quantity of HMGB1 release but inhibited its functional transition seen with priming. In contrast, pan-Nox inhibition (VAS52870) did not induce a functional transition.*

One should bear in mind that the experimental system utilized in **PAPER II** is an artificial system. NLRC4 activation without priming signals *in vivo* is not likely since bacterial infections and concomitant TLR activation is probable. However, several auto-inflammatory disorders such as the cryopyrin-associated periodic syndromes (CAPS), its subtypes and the macrophage activation syndrome (MAS) are associated with gain-of-function mutations in either *NLRP3* or *NLRC4* that in turn trigger spontaneous inflammasome activation [188, 189]. This opens up further possibilities to explore mechanisms regulating HMGB1 function and release regulation in a priming-free inflammasome setting, and the regulation of HMGB1 hyperacetylation by specific inflammasomes. Interestingly, serum HMGB1 from MAS patients contains hyperacetylated NLS regions and diverse functional redox isoforms [190], suggesting that the NLRC4

inflammasome alone may hyperacetylate HMGB1 *in vivo*. Treatment with the apoptotic agent etoposide induced further oxidation of HMGB1 cysteines [190], in agreement with the early HMGB1 cysteine redox observations in apoptotic cells [100]. Whether these patients carried an NLRC4 gain-of-function mutation was not investigated, however, and hence further studies are necessary to interrogate the prevalence of HMGB1 hyperacetylation in such genetic disorders. Comparing HMGB1 acetylation patterns in samples stimulated *in vivo* and *in vitro* revealed striking similarities [49, 179]. Although we only investigated the acetylation pattern in the NLS regions in **PAPER II**, the conserved acetylation pattern of HMGB1 supports the fact that our model may be further utilized to understand this regulation.

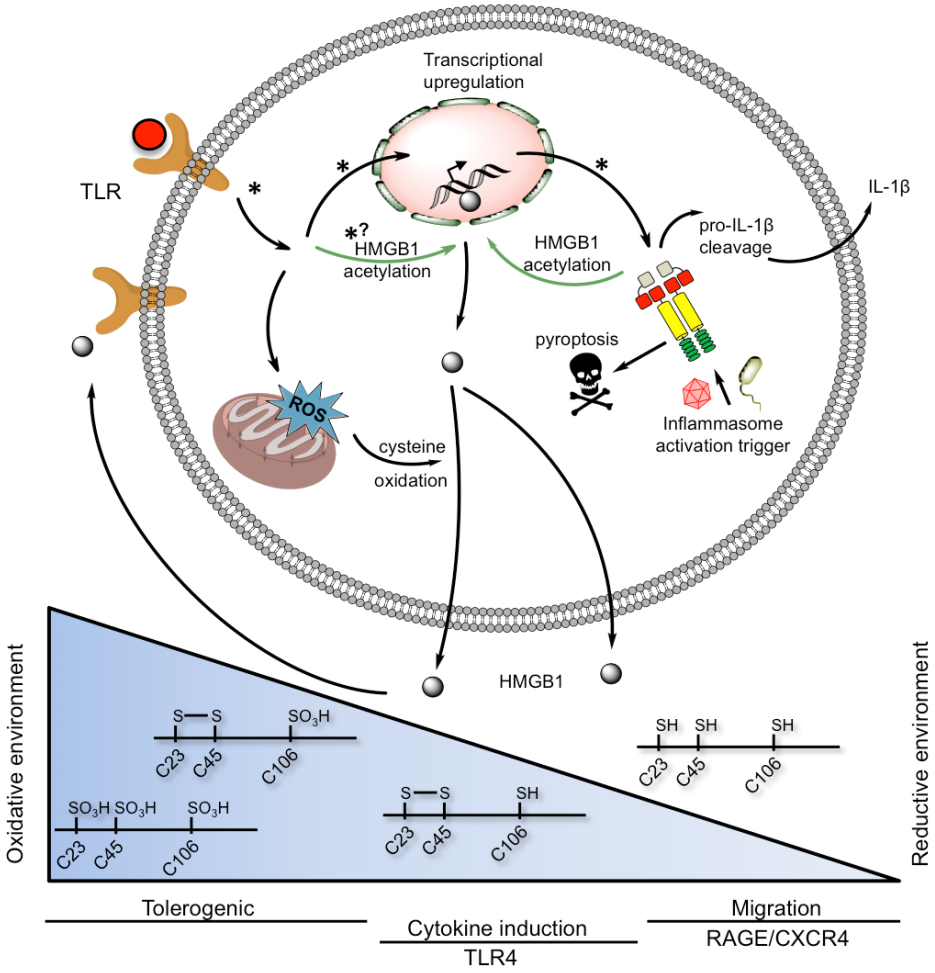

***Figure 6: PTMs regulate the release and function of HMGB1 during inflammasome activation**. NLRC4 inflammasome activation can independently of cell surface TLR activation (priming) induce hyperacetylation that results in cytoplasmic accumulation of HMGB1 and secretion during pyroptosis. Conversely, NLRP3 inflammasome activation and pro-IL-1β requires priming (*****). The priming signal is believed to induce hyperacetylation of HMGB1 prior to release due to NLRP3 inflammasome activation. Cell surface TLR activation induces mtROS production that facilitates a functional transition of HMGB1 by oxidization to the TLR4 activating disulfide isoform.*

Nevertheless, our data provides new insight into the regulation of HMGB1 release and function by inflammasome activation. Our data also clearly suggests that different modes of cell death have the capacity to modulate inflammation, partly by regulating the functional properties of the released HMGB1 and likely also other DAMPs. There is now extensive data of HMGB1 PTMs and how they contribute to the regulation

of HMGB1 function and release in studies of pyroptosis and apoptosis, both of which involve members of the caspase family. Another type of programmed cell death, necroptosis, is independent of caspase activation and is also associated with the release of significant levels of DAMPs, including HMGB1 [191]. Intriguingly, necroptotic cell death is associated with conditions such as I/R injuries [192, 193] and systemic inflammatory response syndrome (SIRS) [194], both being conditions in which HMGB1 has been implicated. Very few variations in the *HMGB1* gene have been recorded [195], and two of these polymorphisms have been reported to affect clinical outcome in critically ill SIRS patients [196]. The release of HMGB1 from macrophages and other cells, without accompanying cell death, has also been demonstrated [50, 68]. In these studies, LPS+IFNγ stimulated macrophages secreted HMGB1 without accompanied cell death and this thus propose release mechanisms independent of canonical inflammasome activation. Interestingly, IFNγ selectively inhibits LPS induced IL-1β production [197, 198] and in addition, selectively inhibits NLRP3 inflammasome activation [199]. Thus, IFNγ could potentially negatively regulate inflammasome activation without affecting HMGB1 release.

The fact that HMGB1 is released as a result of IFNβ stimulation provides an opening for targeting Janus kinase (JAK) and signal transducer and activator of transcription-1 (STAT-1) signaling [179, 200]. Pharmacological inhibition or genetic deletion of JAK/STAT-1 pathway components clearly decreased the release of HMGB1 following LPS stimulation in mouse macrophages [179]. This suggests that JAK/STAT-1 signaling is at least partly responsible for LPS-induced HMGB1 release and that this is an indirect effect of type I IFN production and JAK/STAT1 signaling. Importantly, the significance of STAT-1 signaling in regulation of *in vivo* HMGB1 release is not that obvious and may therefore differ from an *in vitro* setting [200]. In addition, HMGB1 and IL-1α have been proposed to be released by a TRIF-, type I IFN- and caspase-11-dependent mechanism, independently of caspase-1 [201]. Caspase-11 deficiency and administration of HMGB1-specific antibodies provided resistance to experimental sepsis (endotoxemia), whereas lL-1β or IL-18 deficiency does not [17, 19]. This clearly indicates a role of caspase-11 mediated release of HMGB1 during the pathogenesis of sepsis. However, more studies are needed to define potential trans-species cell type similarities or differences.

5.3 HMGB1 as a mediator of chronic inflammation

Several diseases are highly associated with an aberrant extracellular HMGB1 expression. Perhaps one of the most well described chronic inflammatory diseases in which HMGB1 promotes pathogenesis is arthritis. The increased levels of HMGB1 in sera and synovial fluid of RA patients and its putative function as a marker for disease activity have evoked a particular interest for the development of HMGB1-targeted therapies [202]. One has to bear in mind that the determination of total HMGB1 levels by ELISA or by western blotting, does not reveal which HMGB1 isoform is present and hence does not reveal the function of HMGB1 in the disease studied. My group recently reported high levels of HMGB1 in synovial fluid from JIA patients [139]. Although there are clear differences between JIA and RA they also share common features. The functional role of HMGB1 is poorly defined in clinical samples, especially in chronic inflammatory

diseases and has not previously been defined at an isolated site of inflammation. In **PAPER III** we utilized the access to synovial fluid harvested from inflamed joints of 17 randomly selected JIA patients. MS/MS analysis revealed that release of HMGB1 in the inflamed joint was highly associated with hyperacetylation of HMGB1 NLS regions indicative of an active release. Acetylation of HMGB1 has previously been clearly identified in APAP intoxicated patients and HMGB1 blockade in APAP challenged mice is protective [126, 127]. Interestingly, as a biomarker hyperacetylated HMGB1 may be used to predict disease outcome in these patients with high levels being indicative of a poor outcome [126]. Additionally, longitudinal studies clearly suggest that HMGB1 hyperacetylation clearly follows the course of inflammation and is highly associated with inflammatory cell infiltration post-hepatic injury [171].

Neutrophils are the most abundant inflammatory cell type in the joint fluid of patients with arthritis. We could demonstrate a strong correlation between high HMGB1 levels and the presence of a mono-methylation at K43. This PTM has been proposed to be an activated neutrophil-specific modification of HMGB1 [73] and thus suggests that neutrophils contribute to the release of HMGB1 levels in the inflamed joint (**PAPER III**). Unexpectedly, there was no correlation with neutrophil abundance and total HMGB1 levels. However, this does not necessarily reflect the degree of neutrophil activation. Other neutrophil markers such as myeloperoxidase or elastase could potentially provide additional information. SLE patients are associated with an IFN signature, activated neutrophils and the induction of DNA-composed neutrophil extracellular traps (NETs) as a result of a programmed pro- inflammatory neutrophil cell-death (NETosis) [203, 204]. As mentioned, active release of HMGB1 can be induced by type I IFNs and the DNA NETs from SLE patient are known to be decorated with HMGB1 [205]. There is also a NOX activation requirement in order for neutrophils to induce NET formation when stimulated with phorbol 12-myristate 13-acetate (PMA) or bacterial stimuli *in vitro* [206]. Thus, the oxidative burst associated with NET formation could potentially cause a functional transition of HMGB1 in similarity to the effect seen with mtROS production in macrophages (**PAPER II**). In SLE patient sera the levels of HMGB1 can only be reliably measured by western blotting due to immune complex formation and the presence of HMGB1 autoantibodies [207, 208]. In **PAPER III**, HMGB1 levels were measured by ELISA and there is a possibility that this method could give falsely low HMGB1 levels, similar to what has been proven for SLE [170]. Anti-nuclear antibody (ANA) positivity is a common feature in SLE patients and most JIA patients analyzed in **PAPER III** were oligoarticular, a subdiagnosis associated with a higher frequency of ANA [209]. The presence of ANA could potentially indicate an increased frequency of HMGB1 autoantibodies. However, we could not determine any association between levels of HMGB1 and ANA positivity (data not included) and as mentioned earlier (in section 4.3), the frequency of anti-HMGB1 antibodies in analysed samples from our biobank is low (below 2%, Hanna Schierbeck, unpublished data). Nevertheless, one could speculate that the presence of HMGB1 autoantibodies in an analyzed sample could have HMGB1-isoform specificity. If so, that would significantly impact on the outcome of the recorded LC-MS/MS results.

Diverse inflammatory HMGB1 isoforms were recorded in JIA synovial fluid and these variants clearly correlated with active protein secretion as measured by acetylation. Surprisingly, concomitant HMGB1 redox isoforms were present simultaneously in synovial fluid. It may be possible that within the same inflamed joint HMGB1 possesses several functional properties depending on its location, and that its properties/redox status may be regulated by the microenvironment. The presence of extracellular redox regulating systems or changes in pH could possibly affect the reactivity of cysteines. Interestingly, the milieu of synovial fluid is more oxidative than sera from both healthy individuals and patients with joint disorders [210]. Thus other *ex vivo* studies investigating cysteine redox isoforms in sera may not reflect the true functional properties of HMGB1 at the tissue site of inflammation [126, 190].

The small sample size in **PAPER III** does not allow conclusions to be drawn regarding correlation to sub-diagnosis, age, or even gender differences. To allow for such comparisons, the analyzed sample size needs to be increased and is a subject for future studies. Nevertheless, in there appears to be a gender difference with respect to the nature of HMGB1 cysteine redox status (Table 1. in **PAPER III**). In general, synovial HMGB1 isolated from boys displayed more oxidized C23/C45 residues than did HMGB1 isolated from girls. The possible role for ROS and redox-regulating proteins in JIA pathology and the influence of gender is as yet an unknown field. A sex hormone influence on ROS activity is evident [211], but such effects in juvenile patients are obviously highly age-dependent. Interestingly, cells isolated from males are more susceptible to oxidative stress and demonstrate a higher degree of apoptosis [212]. This clearly concords with the relationship of apoptotic cells and more oxidized HMGB1 cysteine isoforms [100].

Moreover, the connection of mtROS production and apoptosis is known [213] and it is likely that mtROS production during apoptosis is responsible for oxidizing HMGB1 cysteines to sulphonyls during apoptotic cell death. MtROS is regulated by several factors and the rate of mtROS production increases with increased cellular oxygen concentrations. There is also a paradoxical increase in mtROS during hypoxic conditions [214] and given the hypoxic environment in synovial fluid [215] it is possible that hypoxia-induced mtROS regulates HMGB1 cysteine oxidation in patients with synovitis.

The use of experimental arthritis models is an important tool in understanding mechanisms of disease and may also be used for preclinical drug development. Several experimental disease models have suggested HMGB1 to be a key mediator of disease pathogenesis, both in arthritis and in other inflammatory conditions. Interestingly, many anti-rheumatic drugs have the capacity to diminish HMGB1 release from monocytes *in vitro* while not being HMGB1-specific [68]. Targeting HMGB1 release or neutralizing extracellular HMGB1 using therapeutic pAbs have previously been demonstrated by my group and by other groups to ameliorate arthritis pathology [132, 133, 159]. The use of pAbs in experimental disease models provides important functional insights into the pathogenesis and may be useful in evaluating the impact of specific antigens, but such treatments are not suitable for human diseases. In contrast, mAbs or humanized mAb variants can be produced using good manufacturing practice (GMP) standards and are thus suitable

for clinical therapeutic applications. Different monoclonal antibodies are currently successfully used as treatments for various human diseases.

In **PAPER IV** we investigated the anti-arthritic activity of a non-commercial monoclonal anti-HMGB1 antibody (2G7) with known therapeutic effects in other inflammatory diseases. In the case of sepsis, 2G7 could be administered up to 24h post-sepsis induction and still confer protection [216]. We evaluated the effect of 2G7 in both induced and spontaneous models of arthritis. CIA is the most widely used arthritis model and in this setting 2G7 treatment was initiated after onset of arthritis in mice with a clinical score of ≥2. This treatment protocol was chosen in order to mimic the clinical reality for anti-rheumatic treatment. Furthermore, due to the aggressive and rapidly progressing nature of this model mice were treated daily with intraperitoneal injections of 2G7 for seven days. Increased serum levels of HMGB1 had been recorded in the spontaneous arthritis model (*DNaseII*$^{-/-}$x*IFN-IR*$^{-/-}$) even before onset of arthritis [159]. Hence when using this model we initiated the 2G7 treatment protocol (every second day for 5 weeks) at 1-2 weeks before predicted onset of arthritis. We could demonstrate that 2G7 treatment significantly improved clinical arthritis severity in both models. The effect of 2G7 was less efficient when compared to anti-TNF treatment (data not included), suggesting that these experimental arthritis models may not be ideal when investigating the therapeutic effects of anti-HMGB1 treatment due to their seemingly high TNF dependence. Although both models share features with clinical RA (and JIA), the validity and clinical relevance of such models in general could be discussed. The effect of 2G7 treatment was for ethical reasons only evaluated during the acute-phase of arthritis, especially in the CIA model. It is possible that HMGB1 perpetuates inflammation at a later and more chronic phase of the disease. This would perhaps be more comparable to clinical arthritis and hence of more clinical significance.

The treatment of human arthritis with TNF blockade is highly beneficial for many patients but not for all. A significant percentage of patients are non-responders and thus there is a need for the development of other targeted therapies [217]. We could confirm high inter- and intra- group variabilities in treatment responses in both these arthritis models, making the interpretation and potential human clinical translation to anti-rheumatic therapy difficult to evaluate. Clinical treatment with anti-TNF antibodies is administered together with methotrexate which synergistically enhance the effect of TNF blockade as compared to treatment with TNF blockade alone [218]. It is possible that such combination treatments would also enhance the clinical effect of 2G7 treatment. Further studies are required to address this issue.

In summary, our results in **PAPERS III** and **IV** together with other reported findings demonstrate that HMGB1 can convey diverse inflammatory functions through active release in the arthritic joint, and that HMGB1 targeted therapies are beneficial in different models of arthritis.

5.4 Forwarding anti-HMGB1 therapy to the clinic

The potential long-term usage of monoclonal antibody-based therapies is strongly influenced by the induction of a human anti-mouse antibody (HAMA) response [219]. Such xenogeneic response reduces the

clinical effect of treatment and might even induce adverse side-effects. The antibody sequence and structure homology between species has allowed genetic engineering of therapeutic antibodies. Hybridization of antigen defining variable regions of mouse antibodies with human constant regions allows for the production of chimeric antibodies less likely to induce a HAMA response while retaining the antigen-specificity and therapeutic effects. Given the wide therapeutic effects of 2G7 (referred to as m2G7 in **PAPER V**) in multiple diseases, biological treatments based on this antibody are possibly of significant clinical importance for treatment of human diseases. In addition, the use of antibodies would allow specific targeting of extracellular HMGB1 while not interfering with intracellular HMGB1 functions. In **PAPER V** we thus generated a human chimeric 2G7 antibody (h2G7) by hybridization with human IgG1. Most clinically approved therapeutic antibodies have an IgG1 backbone in order to retain desired effector functions [220]. IgG1 permits both complement activation and antibody-dependent cell-mediated effects. IgG3 possess similar effector features but the shorter half-life associated with this isotype when compared to IgG1 has likely excluded the development of IgG3-based therapeutics [221]. The substitution of the constant murine domains of m2G7 with human domains did not alter the HMGB1-binding properties nor the specificity.

Given the variability in experimental arthritis models and the seemingly low HMGB1 dependence we opted to investigate the therapeutic efficiency of h2G7 in APAP-induced hepatic inflammation. The pathogenic contribution of HMGB1 to the pathogenesis in this disease has previously been discussed (see section 2.4.3 and 4.2.2). The m2G7 and h2G7 displayed equal therapeutic efficiency in APAP-challenged mice. Interestingly, liver injury was partly inhibited by both 2G7-based treatments and, strikingly, the production of inflammatory mediators was completely abrogated. Post-injury hepatic inflammation is a detrimental event that significantly affects the clinical outcome in APAP overdose patients. Interestingly, doses of h2G7 that did not protect against liver injury still conferred anti-inflammatory effects. This suggests that the effect of HMGB1 is largely mediated via an anti-inflammatory action by blocking HMGB1-induced cytokine production and immune cell infiltration. However, further studies are warranted to investigate whether 2G7 directly or indirectly affects cell migration and cytokine production in this disease model. Additional studies suggest that 2G7 demonstrates equal binding to the HMGB1 cysteine isoforms associated with migration and cytokine induction (**Figure 7**).

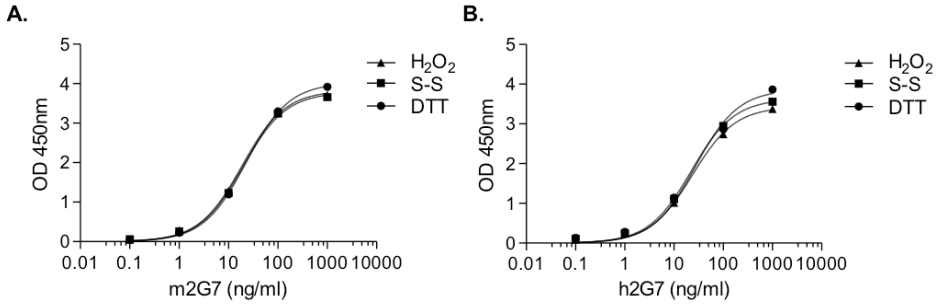

Figure 7: 2G7 binding to HMGB1 is not affected by its redox state in direct ELISA*. TLR4 activating HMGB1 (disulfide HMGB1, **S-S**) was treated with hydrogen peroxide (**H_2O_2**) or **DTT** to generate diverse cysteine redox forms and coated in microtiter plates. No difference in binding with either m2G7 (**A**) or h2G7 (**B**) was recorded.*

A recent study with hepatocyte-specific genetic deletion of HMGB1 indicated that the pathogenic effect of HMGB1 in APAP-induced liver inflammation is mediated via RAGE-regulated neutrophil migration, whereas TLR4 was indispensable in both APAP-exposed and liver I/R-injured mice [129]. In contrast, the detrimental effect of TLR4 activation has been demonstrated in several liver injury models [119, 128, 222].

The effects of immunoglobulins in immunity are not only mediated via the antigen-binding properties but equally important are their crystallizable fragment (Fc)-mediated functions. The mode by which an antibody mediates its therapeutic effects can thus be either by antigen neutralization, steric hindrance, complement activation or ADCC-mediated cell depletion. Antibody engineering of the Fc region has helped in potentiating the therapeutic effect of antibodies, increasing their half-lifes, and importantly defining their mechanisms of action in different diseases [223]. To specifically address the importance of effector functions in 2G7-mediated disease protection we generated variants of h2G7 incapable of binding complement or Fc receptors (FcRs). Variants with an abrogated binding to complement or FcRs displayed similar therapeutic effects as unmodified h2G7 in APAP-challenged animals, suggesting that the effect of h2G7 is not mediated via complement or FcR activation but is likely to be due to 'true' antigen neutralization or by inhibiting HMGB1-receptor interactions via steric hindrance. However, the mechanisms underlying the anti-inflammatory effects of 2G7-based treatments should be addressed in several disease settings, and especially in diseases in which complement- or FcR-mediated effects are of significant importance. Although we could demonstrate a complete abrogation of C1q binding, some antibodies can still retain more than 60% of their complement-dependent cytotoxic activity [224]. Independently of this, if complement activation or FcR mediated effects were of great importance in mediating disease-modulating effects in our system we would have noted distinct therapeutic efficiency with the h2G7 effector function deficient variants. Finally, the generation of a therapeutically active anti-HMGB1 antibody as reported in **PAPER V** is a major advancement towards clinical application of HMGB1-targeted therapy and it also opens up for mechanistic studies of HMGB1 in human disease settings.

6 CONCLUDING REMARKS

In this thesis I have investigated the effects of PTMs in regulation of HMGB1 function and active secretion. Furthermore, I have demonstrated the presence of these PTMs *ex vivo* in a chronic inflammatory disease at the site of inflammation – the arthritic joint. I have also demonstrated the anti-inflammatory therapeutic effects of an anti-HMGB1 specific mAb, in experimental models of arthritis and in a model of chemically-induced liver injury. In addition, I have genetically modified this mAb hence making it more suitable for clinical trials in human disease. The studies in this thesis specifically demonstrate that:

- ✓ HMGB1 cytokine-inducing effects are regulated by cysteine-redox modifications and that the three conserved cysteines (C23, C45 and C106) need to be in defined redox states in order to induce cytokines.
- ✓ Key lysine stretches involved in intracellular localisation and regulation of release are hyperacetylated during NLRC4 inflammasome activation.
- ✓ ROS production resulting from cell surface TLR activation promotes transition from the all-thiol form to the disulfide, cytokine-inducing form of HMGB1.
- ✓ Diverse inflammatory HMGB1 isoforms are simultaneously present in the chronic inflammatory disease JIA and are associated with an active secretion.
- ✓ Blockade of extracellular HMGB1 effects reduce clinical signs of experimental arthritis
- ✓ A humanized version of an anti-HMGB1 mouse mAb retains its therapeutic properties and is not dependent on complement or Fc mediated cytotoxicity.

Many diseases feature an inflammatory signature in their pathogenesis and the extent of inflammation is also in many cases a predictor of outcome. Furthermore, the number of pathological conditions in which inflammatory mechanisms are involved is steadily increasing. Today, biological treatments targeting endogenous inflammatory mediators are used with great success in diverse clinical settings. Many diseases and especially autoimmune chronic inflammatory diseases are, however, multifactorial and thus a significant proportion of patients respond unsatisfactorily to available treatments. There is thus a great need for defining new target molecules in disease states and for development of specific therapies to such new targets. The understanding of HMGB1 and how it contributes to disease pathogenesis has been demonstrated in multiple ways and diseases states, hence highlighting both its central role in the inflammatory response and its potential as a therapeutic target.

Evolution drives biology and proceeds to adapt to the environment over time. The effects of extracellular HMGB1 are hence obviously derived from evolutionary pressure and are not only detrimental, but also of importance for survival. Careful definition of all its functional properties is thus of vital importance and a prerequisite for future development of therapies targeting HMGB1, in order to reduce potential adverse side-effects of such therapies.

The results of my thesis demonstrate how the extracellular functions of HMGB1 are regulated by cysteine redox modifications. By measuring HMGB1 cysteine redox changes one could thus define its function in disease and perhaps it is also possible that specific cysteine redox isoforms of HMGB1 may function as biomarkers to predict disease outcome, similarity to hyperacetylated HMGB1 in APAP-intoxicated patients [126]. However, the complexity of cysteine modifications in comparison to the 'on-off' acetylation of lysines makes cysteine redox unsuitable as biomarkers. Cysteine redox is also more likely to be affected by differences in sample handling in contrast to lysine acetylation. Today there are no available and reliable assays to measure HMGB1 PTMs in large patient cohorts, and for now we rely on a costly and time-consuming approach (e.g. LC-MS/MS). Generation of an antibody assay with both sequence- and PTM-specificity would therefore significantly progress the understanding and contribution of HMGB1 in various disease settings by allowing high throughput analysis in a cost-affordable manner.

Other oxidative cysteine modifications of HMGB1 might occur although none is as yet described. It is likely that such modifications would affect the extracellular function of HMGB1 in a similar manner as the PTMs described in this thesis. The fact that mtROS production can facilitate and functionally regulate HMGB1 by inducing cysteine oxidation would make it interesting to evaluate HMGB1 function in conditions with a mitochondrial dysfunction, such as neurodegenerative disorders or even aging [225, 226]. The intracellular functions of HMGB1 have also, at least partly been suggested to be regulated by cysteine redox. It is likely that more oxidative isoforms, including the TLR4 activating isoform, are overrepresented in such disease states. With the steady increase in age expectancy it is safe to say that neurodegenerative disorders will become even more prevalent and thus the need for new treatments is evident. Besides defining HMGB1 cysteine isoforms in such disorders, generation of HMGB1 cysteine mutant mice would also aid in defining the contribution of various redox isoforms to disease.

The NLRC4 inflammasome has the capacity to induce hyperacetylation of HMGB1 independently of priming *in vitro*, although concomitant activation of multiple PRRs is likely to occur *in vivo* in the case of infection. That priming-independent acetylation occurs *in vivo* might be revealed by studying the subgroup of MAS patients with *NLRC4* gene mutations (encoded by *Ipaf* in mouse cells) that leads to NLRC4 over-activation. Whether other inflammasomes apart from NLRC4 possess the capacity to induce hyperacetylation of HMGB1 in the absence of priming is presently unknown.

Through this thesis work I have advanced the knowledge of the molecular regulation of extracellular HMGB1 effects and of mechanisms involved in active HMGB1 secretion. I have also provided a therapeutic tool, a humanized chimeric mAb specific for HMGB1 (h2G7), that can serve as a basis for further development of a clinical HMGB1-specific treatment option. As compared to endogenous mediators of inflammation such as TNF and IL-1β, the revelation of HMGB1 function and regulation in inflammation is still in an early phase. It is my hope that the work that I present in this thesis supplies important pieces to the puzzle of the endogenous inflammatory mediator HMGB1.

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